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Neuron Glia Biology in Health and Disease Group

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ROLE OF S100B ON CENTRAL NERVOUS SYSTEM DEMYELINATION AND REMYELINATION

Vera Alexandra Padrela Martins Afonso

Dissertação de Mestrado

MESTRADO EM CIÊNCIAS BIOFARMACÊUTICAS

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Dissertação de Mestrado orientada pela Prof.ª Doutora Adelaide Maria Afonso Fernandes Borralho e pela Doutora Andreia Pereira Barateiro

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Aos meus pais.

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I. Introduction

II. Materials and Methods

Abbreviations

ADEM	Acute disseminated encephalomyelitis
AHL	Acute hemorrhagic leukoencephalitis
bFGF	Basic fibroblast growth factor
Ca^{2+}	Calcium
CD	Cluster of Differentiation
cDNA	Complementar deoxyribonucleic acid
CNS	Central nervous system
COSC	Cerebellar organotypic slice cultures
CSF	Cerebrospinal fluid
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GFAP	Glial fibrillary acidic protein
HLA	Human leukocyte antigen
HMGB1	High mobility group box chromosomal protein 1
Iba-1	Ionized calcium-binding adapter molecule 1
IFN-γ	Interferon-γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPC	Lysophosphatidylcholine or Lysolecithin
MBP	Myelin basic protein
MEM	Minimal essential media
mGluR3	Metabotropic glutamate receptor 3
MHC II	Major histocompatibility complex class II
MOG	Myelin oligodendroglial glycoprotein
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cell
NG2	Neural/glial antigen 2
NGF	Nerve growth factor

nM	Nanomolar
NMO	Neuromyelitisoptica
NO	Nitric oxide
NTC	Non-template control
OL	Oligodendrocyte
OPC	Oligodendrocyte precursor cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PLP	Proteolipid protein
PNS	Peripheral nervous system
PPMS	Primary progressive multiple sclerosis
PRMS	Progressive remitting multiple sclerosis
qRT-PCR	Quantitative real-time polymerase chain reaction
RAGE	Receptor for advanced glycation end products
RNA	Ribonucleic acid
ROS	Reactive species of oxygen
RRMS	Relapsing remitting multiple sclerosis
SPMS	Secondary remitting multiple sclerosis
TNF	Tumour necrosis factor
WT	Wild-type
µM	Micromolar

Abstract

S100B is a small Ca^{2+} binding protein member of S100 family. Within the central nervous system, S100B is mostly expressed by astrocytes, though it has been shown to be also expressed by oligodendrocytes, microglia and some neural populations. S100B exerts both intracellular and extracellular functions. Within cells S100B acts as a signaling molecule, being involved in the regulation of energy metabolism and in the modulation of both proliferation and differentiation of neurons and glia. When secreted to the extracellular space, S100B exerts beneficial or detrimental effects in a concentration-dependent manner: (i) nanomolar concentrations have been reported to exert trophic effects whereas (ii) micromolar concentrations have shown to produce neurodegenerative, neuroinflammatory and/or apoptotic outcomes. Multiple sclerosis (MS) is an autoimmune chronic inflammatory disease with a neurodegenerative component, characterized by the occurrence of focal areas of inflammatory demyelination, variable gliosis and relative axonal loss, with limited remyelination. Moreover, data from previous research of other authors and from ongoing work of our group have shown augmented S100B in both CSF, serum and *post-mortem* plaques of MS patients.

With this work we intended to explore the role of S100B in MS main mechanisms. For this end we used mouse cerebellar organotypic slice cultures, a previously described model of demyelination. Therefore, we first evaluated the expression and secretion of S100B in the course of a demyelinating insult. Our results reveal that S100B is released and overexpressed upon demyelination mainly by astrocytes. Next, we also evaluated in what way S100B might affect demyelination, reactivity of glial cells and inflammatory response in consequence of demyelination, by blocking extracellular S100B with a specific antibody. Upon the demyelination insult, we verified an upregulation of myelin proteins and pro-inflammatory cytokines gene expression, as well as activation of astrocytes and microglia. In turn, blockade of S100B appeared to prevent demyelination and reduce both reactive gliosis and expression of pro-inflammatory factors. Altogether, these data strongly suggest that high levels of S100B may exacerbate demyelination and/or delay remyelination.

Keywords: S100B, Multiple Sclerosis, demyelination, inflammatory response

Resumo

A S100B é uma proteína de ligação ao Ca^{2+} da super-família de proteínas S100. No Sistema Nervoso Central a S100B é maioritariamente expressa pelos astrócitos, apesar de haver evidência da sua expressão em oligodendrocitos, microglia e certas populações de neurónios. Sabe-se que o S100B pode ter diferentes funções, consoante a sua localização: intracelular ou extracelular. Quando expresso intracelularmente actua como uma molécula sinalizadora, estando envolvido em processos de regulação do metabolismo energético, bem como na proliferação e diferenciação neuronal e das células gliais. Uma vez secretado para o espaço extracelular, o S100B pode exercer efeitos benéficos ou prejudiciais nas células cerebrais, dependendo da sua concentração: (i) enquanto que concentrações fisiológicas na ordem dos nanomolar tem efeitos tróficos, (ii) concentrações mais elevadas, da ordem dos micromolar, induzem a neurodegeneração, neuroinflamação e apoptose destas células. A Esclerose Múltipla (EM) é uma doença autoimune crónica inflamatória com uma grande componente neurodegenerativa. Patologicamente, a EM é caracterizada pela ocorrência de focos de desmielinização e inflamação, reactividade das células gliais e disfunção neuronal, podendo haver alguma remielinização. Dados de pesquisa publicados por outros autores e dados preliminares do nosso grupo demonstram a presença de níveis anormais de S100B em amostras de líquido cefalo-raquidiano, soro e placas escleróticas de doentes de EM.

Com este trabalho pretendemos investigar o papel da proteína S100B nos principais mecanismos que levam ao desenvolvimento da EM. Para tal, recorremos a um modelo de culturas organotipicas de cerebelo de ratinho, um modelo já usado para estudos de mielinização e desmielinização. Começámos por avaliar os níveis de expressão e secreção de S100B no decurso da desmielinização. De acordo com os resultados obtidos, verificou-se uma sobre-expressão e aumento de secreção de S100B, em resposta ao insulto desmielinizante, mediada maioritariamente pelos astrócitos. Seguidamente decidimos também verificar de que modo o S100B poderá estar a afectar a desmielinização e a reactividade das células gliais e a resposta inflamatória, através do bloqueio extracelular de S100B com um anticorpo especifico para a proteína. Após a desmielinização, observou-se uma marcada desmielinização associada a uma sobre-expressão de genes que regulam as proteínas da mielina e de certas citocinas pro-inflamatórias, bem como a activação dos astrócitos e micróglia. Por sua vez, o bloqueio do S100B extracelular mostrou ter um efeito preventivo da desmielinização bem como atenuante da reactividade glial e da expressão de citocinas

pró-inflamatórias. Estes resultados permitem-nos inferir que níveis elevados de S100B podem favorecer a desmielinização e/ou retardar a remielinização.

Palavras chave: S100B, Esclerose Múltipla, desmielinização, resposta inflamatória

I. Introduction

1. S100B

S100 proteins consist in one of the largest family of small proteins involved in calcium ion (Ca^{2+}) homeostasis. Members of the S100 protein family are known to contain two EF-hand helix-loop-helix Ca^{2+} binding domains: a canonical C-terminal and a non-canonical N-terminal (Kawasaki et al., 1998). Ca^{2+} binds to these domains causing conformational changes, exposing a hydrophobic cleft, which is required for interaction with target proteins and their modulation (Yap et al., 1999; Zimmer and Weber, 2010).

S100A1 and S100B were the first members of S100 family to be identified as components of the S100 protein fraction that was extracted from bovine brain, this fraction presented homodimers and heterodimers of both proteins (Moore, 1965; Moore et al., 1968). S100B is expressed by a restrict number of cell types from different organs and tissues, although it appears to be abundantly expressed by central and peripheral nervous system glial cells (Donato, 2003; Rambotti et al., 1989). In these cells S100B may be observed both in a soluble form diffusely in the cytoplasm or associated with intracellular membranes, centrosomes, microtubules and type III intermediate filaments (Donato, 2003, 2007).

1.1. S100B double life

As most S100 proteins, S100B exists within cells as antiparallel homodimers, with the two subunits held together by non-covalent disulfide bonds (Donato, 2001;

Heizmann, 2002) or as an heterocomplex in association with S100A1 monomer (Isobe et al., 1983).

Recent studies have shown that S100B monomers have the ability to assemble into more complex oligomers, such as tetramers, hexamers and octamers, and that both the degree of oligomerization and the type of bond that holds the monomers might be related to its function (Donato, 2007). Dimeric forms of S100B are mainly associated with intracellular roles, such as modulation of microtubule assembly and regulation of cell cycle by interaction with transcription factors in a Ca^{2+}-dependent manner (Davey et al., 2001). Therefore, changes in intracellular Ca^{2+} concentrations cause S100B secretion to the extracellular space where it is known to exert cytokine-like functions (Davey et al., 2001). Such functions require specific binding to cell receptors such as the receptor for advanced glycation end products (RAGE), to which S100B binds preferentially when in tetrameric/octameric forms promoting RAGE dimerization (Ostendorp et al., 2007).

RAGE receptor is a member of the immunoglobulin-like cell surface receptor super family that is primarily involved in inflammation, nephropathy, neurodegeneration, and cancer. It is composed by an extracellular moiety, with one N-terminal V-type and two C-type Ig domains, a single transmembrane spanning helix and a short cytosolic domain, which is necessary for signal transduction (Neeper et al., 1992; Rauvala and Rouhiainen, 2010; Soroka et al., 2003). The outcome of S100B binding to RAGE depends on the extracellular levels of the ligand: nanomolar (nM) concentrations of S100B were shown to exert neurotrophic and neuroprotective functions at neuronal level, promoting neurite growth and neuronal survival (Barger et al., 1995; Kögel et al., 2004); and micromolar (μM) concentrations have been shown to accomplish opposite effects, activating a pro-apoptotic pathways (Van Eldik and Wainwright, 2003; Huttunen et al., 2000). Recent studies have shown that extracellular S100B does not operate solely via RAGE binding. It was suggested that in proliferating myoblasts S100B binds to the cell fibroblast growth factor (FGF) receptor 1 (FGFR1) trough interaction with basic FGF (bFGF) (Riuzzi et al., 2011). This engagement results in the recruitment of RAGE into a RAGE/S100B/bFGF/FGFR tetracomplex, which in turn will enhance the

mitogenic signaling pathway activated by bFGF-FGFR1 assembling and inactivate RAGE signaling, promoting skeletal muscle regeneration (Riuzzi et al., 2012; Sorci et al., 2013).

As previously mentioned, within cells S100B might act as an intracellular regulator. Indeed, upon Ca^{2+} binding S100B dimmers suffer a conformational change exposing the hydrophobic residues, and consequently creating a hydrophobic cleft through which S100B can bind to target proteins (Iuvone et al., 2007). Many target proteins of S100B have been identified, involving S100B in diverse intracellular events as regulation of Ca^{2+} homeostasis, cell proliferation and differentiation, protein phosphorylation, enzyme and channel activities, protein degradation, assembly of several cytoskeleton components, cell locomotion, dark adaptation of photoreceptors and the innate inflammatory response (Table I. 1). It is quite clearly that S100B plays a protective role as long as it is kept within the cells at physiological levels.

Within the Central Nervous System (CNS), S100B is known to be actively secreted by astrocytes, which is believed to occur through activation of metabotropic glutamate receptor 3 (mGluR3) in a neural and synaptic activity dependent manner (Sakatani et al., 2008; Shashoua et al., 1984). Once in the extracellular space S100B exerts beneficial or detrimental effects on neurons, microglia and astrocytes depending on the concentration (Van Eldik and Wainwright, 2003). At physiological concentrations around nM levels S100B behaves as a signaling trophic protein promoting neuronal survival, astrocytic proliferation and microglia quiescence (Gonçalves et al., 2000; Reali et al., 2005; Selinfreund et al., 1991; Zhang et al., 2011b).

When S100B levels rise to the range of μM the whole scenario is reversed with production of reactive species of oxygen (ROS) leading to neuronal death. Moreover, inflammatory activities of astrocytes are potentiated and microglia is activated as evidenced by the expression and release of pro-inflammatory cytokines (Adami et al., 2001; Businaro et al., 2006; Petrova et al., 2000). Several studies have shown that both trophic and toxic effects of extracellular S100B are mediated by its binding to

RAGE and might depend on the intensity and extent of activation of the receptor, as well as on its level of expression in CNS cells (Donato, 2007).

Table I. 1 – S100B intracellular main target proteins and respective functions.

Cell Function	Target Protein/ Pathways	Reference
Cell proliferation and differentiation	p53	(Lin et al., 2004)
	IKKβ/NF-κB	(Tubaro et al., 2011)
	PI3-K/Akt/$p21^{WAF1}$/cdk4/Rb/E2F	(Arcuri et al., 2005)
Receptor function	D2 receptor/$ERK_{1/2}$/adenyl cyclase	(Liu et al., 2008)
Protein phosphorylation	τ proteins	(Baudier and Cole, 1988)
	GAP43	(Sheu et al., 1994)
	p80	(Pozdnyakov et al., 1998)
	p53	(Baudier et al., 1992)
	GFAP and vimentin	(Ziegler et al., 1998)
Ca^{2+} homeostasis	AHNAK	(Gentil et al., 2001)
	Annexin 6	(Marisa et al., 1998)
Protein degradation	E3 ligase hdm2	(Wilder et al., 2006)
Cell locomotion	Src/PI3-K/RhoA/ROCK and Src/PI3-K/Akt/Gsk3β/Rac1	(Brozzi et al., 2009)
Enzyme activity	Membrane bound GC	(Rambotti et al., 1999)
	Fructose-1,6-bisphospate aldolase	(Zimmer & Van Eldik, 1986)
Cytoskeleton assembly	Microtubules	(Donato, 1984)
	Type III intermediate filaments	(Garbuglia et al., 1999)
	Caldesmon	(Polyakov et al., 1998)
	Calponin	(Fujii et al., 1994)
	IQGAP1	(Mbele et al., 2002)
Transcription regulation	p53	(Lin et al., 2001)
Channel activity	EAG1 K^+	(Sahoo et al., 2010)

Nevertheless, although having been extensively studied over the past five decades, there is no evidence of accordance between intracellular regulatory activities of S100B and its extracellular effects; that is, no unitary theory of intracellular and extracellular S100B effects can be currently predicted.

1.1.1. Intracellular functions of S100B in the Central Nervous System

It is now known that within CNS S100B is mostly expressed by astrocytes and some oligodendrocytes (OL) of hippocampus and brain cortex, although it can also be present in certain neuronal subpopulations (Gerlach et al., 2006; Hachem et al., 2005; Steiner et al., 2007). The greatest amount of S100B is located within the cytoplasm, with only about 7% bound to the membranes. S100B involvement in cellular events has been extensively studied and includes the regulation of cell proliferation and differentiation, as well as the control of the assembly of cytoskeleton components, intracellular Ca2+ homeostasis and proteins activities, interactions and modifications (Figure I. 1) (Donato et al., 2009).

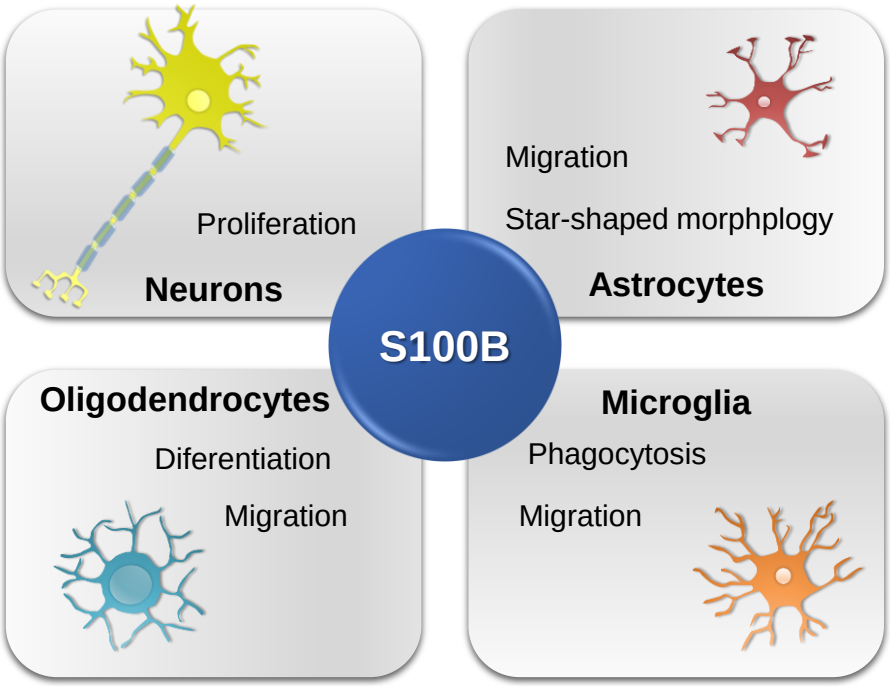

Figure I. 1 – Schematic representation of S100B intracellular effects in central nervous system cells.

It is known that S100B interacts with cytoskeleton components, modulating their assembly and disassembly (Garbuglia et al., 1998; Sorci et al., 1998). Indeed, recent data have shown that S100B is involved in astrocytic migratory ability and star-shaped morphology by regulating the molecular organization of actin filaments (F-actin) in a PI3-K dependent manner. PI3-K regulates then Akt(PKB)/GSK3β/Rac1 and small GTPase RhoA/ROCK pathways, both involved in the formation of stress fibers

promoting the creation of cytoplasmic extensions and favoyring cell locomotion (Brozzi et al., 2009; Watanabe et al., 2004). In addition, S100B was found to be associated with microtubule-like structures and centrosomes on microglia, suggesting its involvement on activation and phagocytic ability characteristics of these glial cells (Adami et al., 2001).

S100B was shown to play an important role in the regulation of neuronal proliferation and differentiation during early development stages. The overexpression of S100B in the neuronal cell line pheochromacytoma PC12 exposed to the neurotrophin nerve growth factor (NGF) has been demonstrated to enhance cell proliferation and reduce differentiation through activation of Akt/$p21^{WAF1}$/cyclin D1-cdk4/Rb/E2F pathway (Arcuri et al., 2005). It has also been shown that S100B is involved in the regulation of astrocytes proliferation and differentiation, trough activation of PI3-K in a Src kinase-dependent manner (Brozzi et al., 2009). Experiments in mouse cerebellar cultures have demonstrated that S100B plays a role in the maintenance of Ca^{2+} homeostasis in astrocytes which in turn are important in the regulation of neurophysiology and in the modulation of neuronal activity (Agulhon et al., 2008; Xiong et al., 2000).

Moreover, recent data regarding oligodendroglial cells revealed the presence of an expression pattern of S100B along their differentiation and maturation (Figure I. 2) (Deloulme et al., 2004). Mature myelinating OL are known to descend from a pool of multipotent precursor cells maintained in specific regions of the CNS. These precursor cells undergo several changes at morphological and antigenic markers expression levels, until becoming myelinating OL, being described four transitional cell stages: OL precursor cells (OPC), preoligodendrocytes (or late OPC), immature (or pre-myelinating) OL and mature (or myelinating) OL (Dawson et al., 2000; Levine et al., 2001).

Particularly, S100B is mostly expressed in these intermediate cell stages, suggesting its enrolment in OPC proliferation and differentiation into mature OL. Also, S100B expression was shown to greatly increase during the transition between non-myelinating pre-OL and mature OL, which might be explained by the fact that

cytoplasmic S100B is associated with cytoskeleton components, such as microtubules, present in the extensions and membranous sheaths developed by maturing OL (Deloulme et al., 2004; Zhang, 2001).

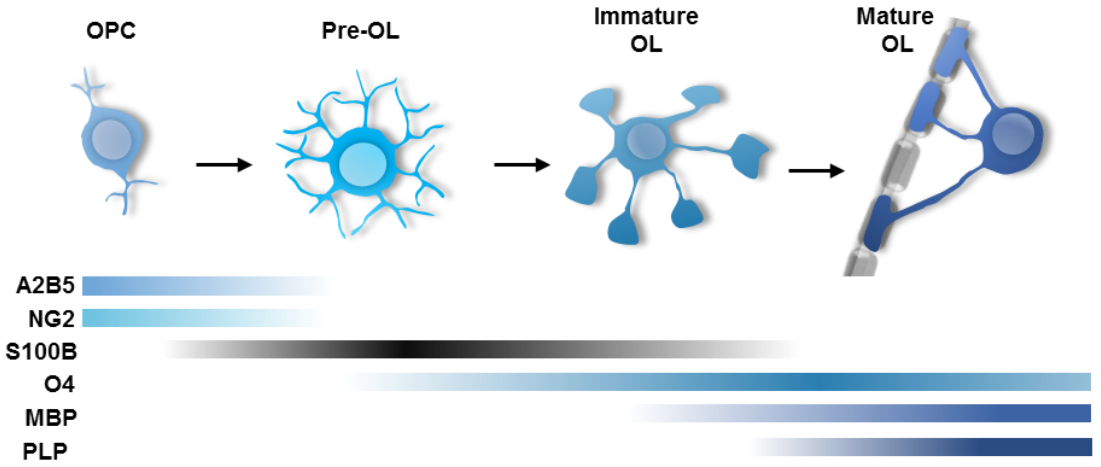

Figure I. 2 – Schematic representation of S100B and other markers expression along OL lineage development. OPC, oligodendrocyte precursor cells; OL, oligodendrocyte; NG2, chondroitin sulphate proteoglycan; MBP, myelin basic protein; PLP, proteolipid protein, (Adapted from Deloulme et al., 2004; S. C. Zhang, 2001)

All this regulation events carried out by S100B makes evident its importance along the CNS development, as well as in the course of brain insults when astrocytes and microglia become activated and, together with OPC, migrate to areas of insult in an effort to restore its proper performance (Zlokovic, 2008).

1.1.2. Extracellular functions of S100B in the Central Nervous System

As previously mentioned, within CNS S100B is mainly secreted by astrocytes in a constitutive manner (Shashoua et al., 1984). Secretion of S100B has been shown to be regulated by a number of external factors and condition, some of them augment it others repress it. Indeed, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), serotonin, lysophosphatidic acid, low levels of glutamate, low extracellular Ca^{2+} levels, metabolic stress and serum deprivation are known to enhance S100B release. On the other hand, factors as elevated levels of glutamate and glucose, inhibition of Src kinase

activity, cell confluence, Ca^{2+} channel blockade and gap junction inhibition are known to reduce S100B secretion (Sorci et al., 2010).

Interestingly, depending on the concentration present in the extracellular space, S100B is known to act as a neurotrophic or neurotoxic molecule (Figure I. 3). Indeed, under physiological conditions, S100B levels in the brain extracellular space range around a few nM concentrations. At this concentration S100B is known to exert trophic effects on neurons, stimulating neurite outgrowth and increasing neuronal survival during development and following injury via stimulation of extracellular signal-regulated kinases (ERK) 1 and 2 ad of the nuclear factor-κB (NF-κB) as well as up-regulation of the anti-apoptotic protein Bcl-2 (Barger et al., 1995; Businaro et al., 2006; Huttunen et al., 2000). Still, under stress conditions S100B tends to be secreted in higher amounts, reaching concentrations on the order of μM, at which S100B exerts neurotoxic effects via overproduction of reactive oxygen species and activation of apoptotic pathways (Donato et al., 2009).

As described above, both trophic and toxic effects of extracellular S100B are mediated in the brain by binding to the receptor RAGE (Donato, 2007; Leclerc et al., 2009). RAGE is expressed in several cell types, including neurons, and its ligation by S100B on these cells has been shown to be someway responsible for the protein dual roles (Sorci et al., 2013). Low S100B doses protect neuronal cells from β-amyloid toxicity and also activates anti-apoptotic pathways via RAGE engagement. In contrast, an excessive stimulation of RAGE as consequence of S100B binding results in the hyper-activation of the pro-apoptotic Ras/MEK/ERK pathway and subsequent overproduction of reactive oxygen species (Businaro et al., 2006; Huttunen et al., 2000). Nevertheless, opposite outcomes resultant from S100B/RAGE interaction might depend on the number of RAGE molecules available on the cell surface. Moreover, since only high levels of S100B are able to promote RAGE dimerization consequent activation of pro-inflammatory cells, it is possible that, under low levels of S100B, RAGE may recruit other factors to different intracellular signaling pathways (Sorci et al., 2010, 2013).

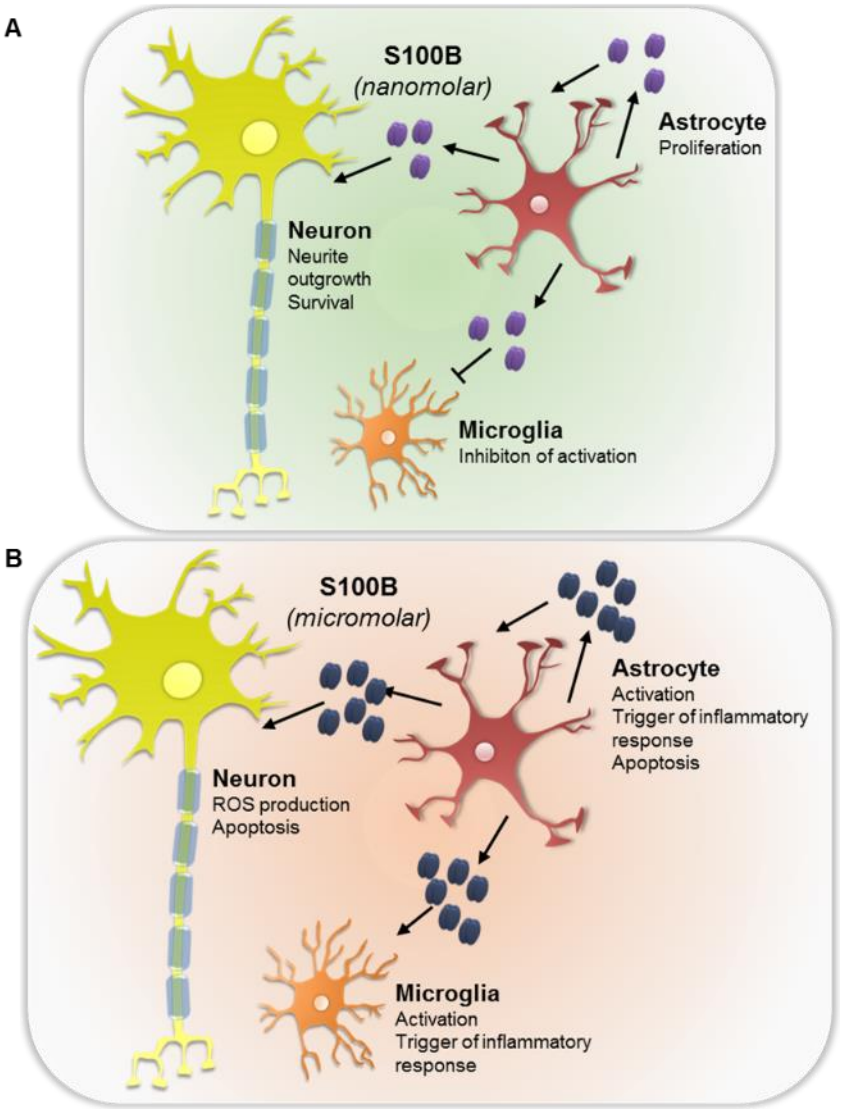

Figure I. 3 – Schematic representation of S100B extracellular effects on central nervous system cells. At nanomolar concentrations S100B exerts a beneficial effect on CNS cells (A), whether higher concentrations are detrimental, favoring a pro-inflammatory scenery (B).

Besides neurons, extracellular S100B also affects surrounding glial cells, mainly astrocytes and microglia, in a concentration-dependent manner (Nardin et al., 2007). S100B acts on astrocytes in an autocrine way, and at nM levels stimulates their proliferation by phosphorylation of ERK1/2 (Gonçalves et al., 2000; Selinfreund et al., 1991). This astrogliosis is counteracted by elevated concentrations of S100B, that stimulate inducible nitric oxide synthase (iNOS) activity, via activation of NF-κB and nitric oxide (NO), once synthesized leads to astrocyte apoptosis (Lam et al., 2001; Petrova et al., 2000). Similarly, high S100B stimulates IL-1β, interleukin-6 (IL-6) and TNF-α release from astrocytes, meaning that S100B is likely to take part on activation of brain inflammatory response (Hu and Van Eldik, 1999). Controversially, high S100B

levels might be beneficial in the course of brain inflammatory processes, once by inducing apoptosis might contribute to the reduction of activated astrocytes which in turn might attenuate inflammatory response.

Extracellular S100B also affect microglia, the brain resident macrophages (Adami et al., 2001). At physiological levels, S100B has the effect to prevent microglia activation via STAT3 pathway (Zhang et al., 2011b). By contrast, μM concentrations of S100B synergistically with cofactors like bacterial endotoxin or interferon-γ (IFN- γ) are known to mediate microglia activation. This activation occurs via stimulation of iNOS and consequent enhance of NO release, being crucial to trigger brain inflammatory response (Adami et al., 2001; Petrova et al., 2000). Some effects of S100B on both astrocytes and microglia also seem to be mediated by RAGE ligation (Ponath et al., 2007; Sorci et al., 2013). Thus, extracellular S100B is now being used as a parameter of glial activation or commitment in several situations of brain injury.

S100B expression and release was thought to be restricted to astrocytes for decades. However recent data has demonstrated that mature OL from the OL-93 cell line also secrete S100B at a higher level than astrocytes under serum and glucose deprivation conditions (Steiner et al., 2008a). Consequently, such high concentrations of S100B might be detrimental for both OL proper function and OPC differentiation into myelinating OL. In this regard, our group observed that exposure of OPC to pathological concentrations of S100B (1μM) decrease OPC differentiation when compared to OPC treated with control or S100B physiological levels (10 nM), impairing therefore their ability to became myelinating cells (Figure I. 4; unpublished data). These results suggest a possible enrolment of S100B in myelination.

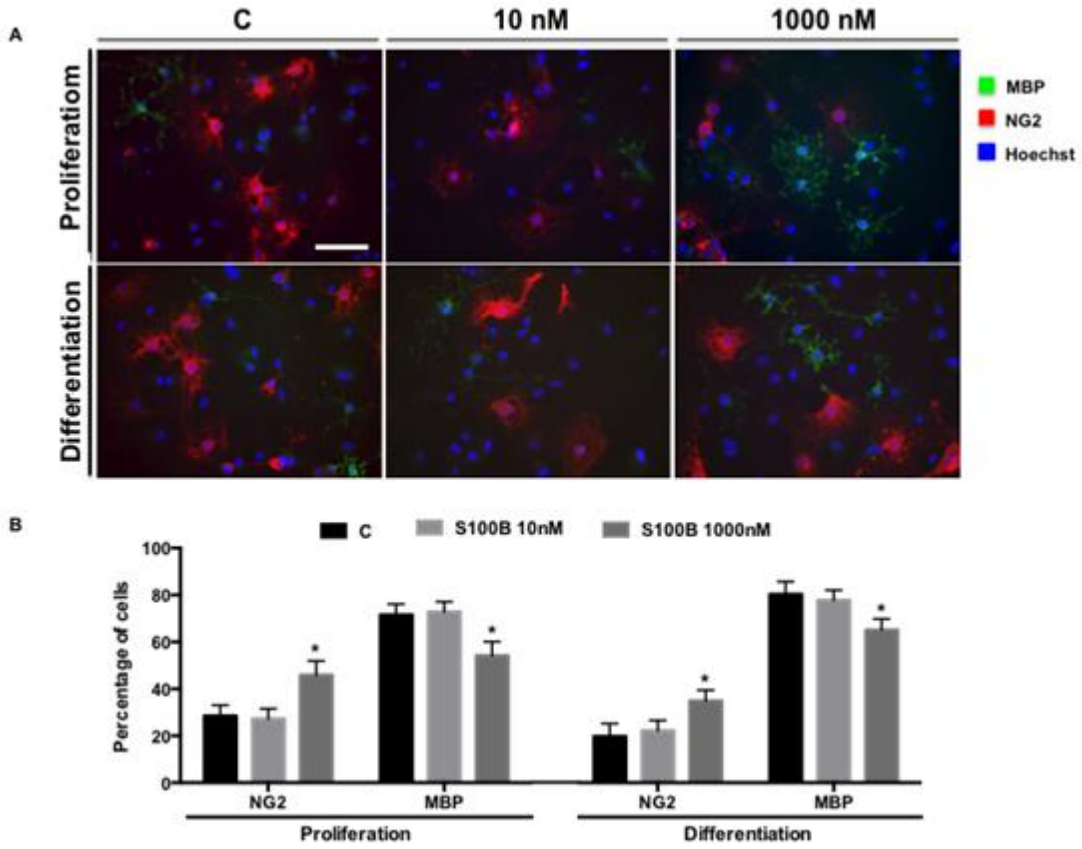

Figure I. 4 – Extracellular effects of different S100B concentrations upon oligodendrocyte precursor cells (OPC) development. Only low doses of S100B reduce the number of myelin basic protein (MBP)+ cells (red), slightly increasing the number of NG2+ immature ones (green). (Adapted from Santos G PhD work).

1.2. S100B as a biomarker of brain damage

There are several studies that reveal an association between elevated levels of S100B and CNS disorders, based both on S100B detection on body fluids for post-mortem specimens. Augmented S100B levels were first detected in cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients in the acute phase (Michetti et al., 1979). Since then, studies of S100B detrimental effects were extended to other diseases of the CNS. High levels of S100B have been detected in a number of brain-related diseases such as Alzheimer disease, Down syndrome, amyotrophic lateral sclerosis and Parkinson disease (Harpio and Einarsson, 2004; Shakeri et al., 2012; Steiner et al., 2011; Uher and Bob, 2012).

Actually, due to high levels of S100B expression in the brain and because it is released from injured astroglial and/or OL cells in the course of an insult, S100B is increasingly being considered as a biomarker for brain damage (Shakeri et al., 2012; Sun and Feng, 2013). Indeed, S100B has already been identified as a potential biomarker in cases of premature and traumatic brain injury, once it has been reported to be augmented in the CSF and serum of patients following such traumas (Beharier et al., 2013; Kleindienst and Ross Bullock, 2006).

Moreover, the fact that some of the detrimental effects resulting from overexpression of S100B are mediated trough RAGE engagement and that both astrocytes and microglia express this receptor, suggest a possible involvement of S100B/RAGE interaction in the course of some neurodegenerative disorders (Bianchi et al., 2011; Donato et al., 2009; Ponath et al., 2007). In fact, recent data from a human neuroblastoma cell line (LAN-5) suggest that while nM levels of S100B counteract amyloid-β (Aβ) peptide neurotoxic effects in a RAGE dependent manner, μM levels S100B exacerbates Aβ toxicity (Businaro et al., 2006).

Overall, given the importance of S100B in the regulation of several events within CNS cells as well as its implication in some neurodegenerative diseases, it is of great interest to explore its possible role in other brain damage related pathologies.

2. Demyelinating Disorders

A demyelinating disease consists of total or partial loss of the myelin sheath that surrounds axons, compromising their proper function and integrity. This loss occurs in response to intrinsic or external factors, such as inflammatory processes, metabolic derangements, hypoxia-ischemia and viral infections, that might target either the own myelin or the cells that synthesize it – OL in the CNS or Schwann cells in the peripheral nervous system (PNS) (Love, 2006; Mayo et al., 2012). Usually, following demyelination spontaneous remyelination might occur allowing a partial recovery, and it is the balance between demyelination and remyelination that defines the outcome of the disease (Franklin and Ffrench-Constant, 2008).

CNS demyelinating disorders include MS, Marburg disease, neuromyelitisoptica (NMO), Balo's concentric sclerosis, acute disseminated encephalomyelitis (ADEM) and acute hemorrhagic leukoencephalitis (AHL) (Bunyan et al., 2012; Popescu and Lucchinetti, 2012). Since MS is the most prevalent demyelinating disorder among young adults we focused our study is this disorder.

2.1. Multiple Sclerosis as the most common demyelinating disease

MS is the major demyelinating disease of the CNS, occurring in an inflammatory background. MS is considered to be the most common cause of non-traumatic disability in young adults, with a great socio-economic impact worldwide, with each individual costing about €2 million during his lifetime. Most recent data show that there are about 2.5 million people with MS worldwide, with an incidence of 2.5 per 100 000, and a higher prevalence in developed regions (e.g. Europe with 80/100 000) than in underdeveloped countries (e.g. Africa with 0,3/100 000) (WHO, 2008). In Portugal, accordingly to the most recent studies there is an estimated prevalence of 56,2 per 100 000, i.e., there are 5620 MS patients per 10 million inhabitants, of which only about 3500 to 4000 are known to receive directed therapy for the disease (de Sá et al., 2012).

MS is characterized by the occurrence of areas of acute focal inflammatory demyelination, variable gliosis and relative axonal loss with limited remyelination, culminating in the formation of chronic multifocal sclerotic plaques. These lesions occur preferentially in optic nerves, subpial spinal cord, brainstem, cerebellum, and periventricular white matter regions (Compston and Coles, 2008; Love, 2006). MS pathogenesis is not fully understood and even though it is subject of controversy, there are strong evidence for an autoimmune pattern with auto-reactive immune cells crossing the blood-brain barrier to attack myelin and axons (Brassat, 2012; Corthals, 2011).

The onset of MS occurs between 15 and 55 years and is two to three times more frequently in women than men. MS etiology is unknown, though several studies for the

past few years have shown that it results from the interaction of diverse risk factors such as: genetic susceptibility (MHC class II-associated HLA-DRB1*15, ethnic origin and sex), viral infection (EpsteinBarr virus), behavior (smoking) and environment (latitude and lack of vitamin D) (Ramagopalan et al., 2010). Symptomatically, MS is characterized by the occurrence of motor weakness, vision loss, diplopia, ataxia, cognitive impairment, bowel and bladder dysfunction and cortical dysfunctions such as seizures (Compston and Coles, 2008; Milo and Miller, 2014).

2.1.1. MS clinical course

MS clinical course has been categorized in four types, accordingly to the emergence of disabilities (Figure I. 5). Most frequently, MS disease starts with a course of demyelination insults followed by periods of neurological recovery, being designated relapsing-remitting MS (RRMS). This revisable disability is characterized by focal areas of inflammatory demyelination in which myelin, OL and axons become damaged (Hanafy and Sloane, 2011; Peterson and Fujinami, 2007). Relapses are associated with the reactivation of old lesions or even with the appearance of new ones, while remittance periods are the result of the resolution of inflammation and remyelination (Chandran et al., 2008; Compston and Coles, 2008). The time elapsed between relapses is variable, with the latent phase between the first manifestation of MS and the first relapse going from little months to several years. With the recurrence of relapsing and remittance episodes, recovery from each episode tends to be incomplete with consequent accumulation of persistent symptoms (Compston and Coles, 2008; Trapp and Nave, 2008).

RRMS stage is normally followed by a phase of uninterrupted disease progression, determined as secondary progressive MS (SPMS). This phase is characterized by a continuous and irreversible neurological degeneration, as there is no remyelination and subsequently no axonal regeneration (Vukusic and Confavreux, 2003). A small percentage of MS patients (10-15%) doesn’t pass by the relapsing-remitting phase, but instead experiment an uninterrupted disease progression from the beginning, being

referred as primary progressive MS (PPMS). The rarest type of MS is progressive-relapsing MS (PRMS) characterized by a progressive onset interspersed by acute relapses, with or without recovery, and a continuous progression in the period between the relapses. PRMS may represent a rare subtype of PPMS since both present a similar history (Lim et al., 2004; Wolinsky, 2003).

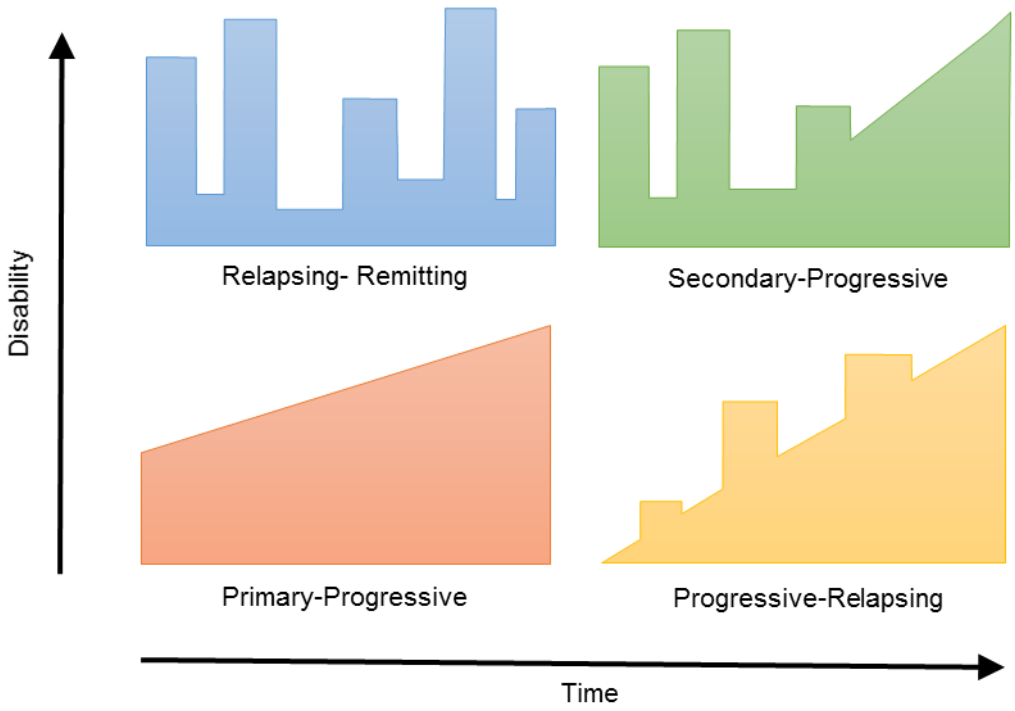

Figure I. 5 – Schematic illustration of how of disability evolves through time in MS.

Progressive forms of MS are the most troublesome, not only because patients become disabled due to fail of remyelination but also because there is no successful therapeutic at the time to combat physical, cognitive, and quality of life deterioration that these MS patients have to face. Thus, it is imperative to research and develop novel therapies in this direction.

2.1.2. MS pathophysiology

The main hallmark of MS is the loss of myelin. The main function of myelin sheaths is to enable saltatory conduction of impulses, playing a dynamic and vital role on the

maintenance of axonal homeostasis and integrity (Compston and Coles, 2008; Griffiths, 1998). Within the CNS, myelin is synthesized by mature OL that emit processes from their own cytoplasmic membranes and continuously envelop axons, forming a multilamellar compacted membrane (Deber and Reynolds, 1991). As extensions of cytoplasmic membranes, myelin sheaths consist on a proteolipidic foundation and are constituted by two major proteins: proteolipid protein (PLP) and myelin basic protein (MBP). Unlike PNS, where each myelin sheath is produced by a single Schwann cell, in the CNS OL are able to furnish about 40 or more adjacent axons, influencing axonal caliber and improving their integrity and stability (Tzakos et al., 2005; Witt and Brady, 2000).

Although immune-mediate theory of MS pathology is not the only under discussion, it is the one that is most described and has been target of extensive research (Corthals, 2011; Nakahara et al., 2012). In this context, the loss of myelin sheaths occurs as consequence of the migration across the blood brain barrier of auto-reactive T-lymphocytes against myelin components. These activated T-cells will be induced to differentiate in lymphocytes T helper CD4+ (ThCD4+) which in turn will be reactivated by MHC-II-expressing macrophages or dendritic cells that present myelin elements (Figure I. 6) (Mayo et al., 2012).

In turn, reactivated T-cells circulate within the CNS and together with activated macrophages, microglia and astrocytes secrete soluble factors like pro-inflammatory cytokines and chemokines, such as IFN-γ, TNF-α, high motility group box 1 (HMGB1) and matrix metalloproteinases (MMPs) (Amor et al., 2010; Van der Walt et al., 2010). The accumulation of these pro-inflammatory factors as well as the cells that produce them leads to the recruitment of naïve microglia and consequent activation, amplifying the inflammatory and immune response that culminates with demyelination (Lassmann and van Horssen, 2011; Nakahara et al., 2012).

A growing body of evidence strongly suggests the involvement of inflammasomes in the regulation of inflammatory response that leads to demyelination. NLRP3, a member of the nucleotide-binding domain, leucine-rich repeat containing (NLR) family,

is pyrin-domain containing 3, is a core component of the inflamasome complex (Lequerré et al., 2007; Sutterwala et al., 2006). Moreover, NLRP3 has been suggested to be implicated in neuroinflammation and demyelination events in an *in vivo* model of MS trough interleukin-18 (IL-18). The same study has revealed that IL-18, one of the end products of the inflammasome, exacerbates demyelination and inflammatory outcomes, such as astrocytic and microglia activation (Jha et al., 2010).

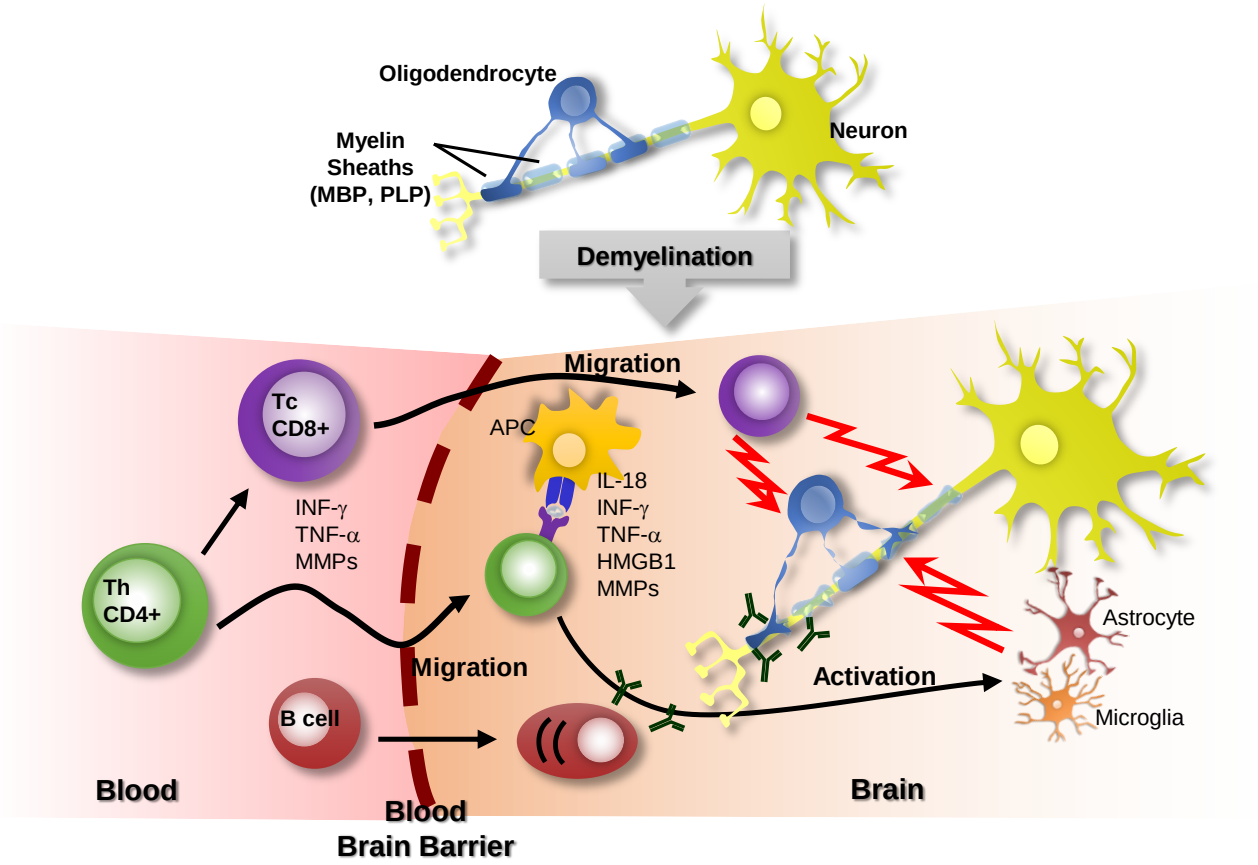

Figure I. 6 – Schematic representation of MS pathophysiology mechanisms. Autoreactive T CD4+ cells are activated in the periphery, transmigrate trough the blood-brain barrier (BBB) into the central nervous system (CNS), and are locally reactivated by antigen presenting cells (APS). Activated T CD4+ cells secrete pro-inflammatory cytokines and, together with other successively recruited and activated immune cells, such as T CD8+ and B cells, create a pro-inflammatory environment, leading to myelin, oligodendrocyte (OL) and axon damage, with consequent neurologic dysfunction. MBP, myelin basic protein; PLP, proteolipid protein; Tc, T cytotoxic lymphocytes; Th, T helper lymphocytes; APC, antigen presenting cell; IL-18, interleukin-18; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor γ; HMGB1, high motility group box 1; MMP, matrix metalloproteinase.

Another important factor in the potentiation of inflammation is HMGB1, which within the CNS can be secreted by astrocytes, microglia and neurons (Andersson et al., 2000). HMGB1 triggers inflammatory response mainly through binding to RAGE, the same receptor by which extracellular S100B operates (Kokkola et al., 2005). Recent studies have shown an exacerbated expression of both HMGB1 its receptor RAGE and in MS lesions, suggesting a potential interaction of these molecules in the inflammatory process involved in MS pathogenesis (Andersson et al., 2008).

Following demyelination myelin debris are phagocytized by local macrophages, differentiated from monocytes that cross the blood-brain barrier (BBB), and from resident microglia. Additionally, astrocytes and microglia secrete neurotrophic molecules that mediate the recruitment of OPC to the freshly demyelinated focuses. Once in MS lesions, OPC are induced to proliferate and differentiate into pre-OL, pre-myelinating OL, and then mature myelinating OL. These cells will then regenerate myelin sheaths, restoring axonal conduction in some way (Miron et al., 2011). This, remyelination, is as effective as sooner migration of OPC occurs, i.e., it has been shown that remyelination might not succeed in case of a late migration of OPC or failure to differentiate. With the progression of the disease and the exposure to repeatedly demyelinating insults, OPC pool undergoes exhausted and remyelination also tends to fail (Chandran et al., 2008; Franklin and Ffrench-Constant, 2008; Patel and Klein, 2011).

2.2. S100B in Multiple Sclerosis

As mentioned above, MS pursues a path of neuroinflammation, glial reactivity and oligodendropathy. Similarly, S100B has been shown to be involved in the previous events in some way. In fact, it has been shown the presence of S100B in acute lesions of *post-mortem* brain tissue of MS patients in the relapsing remitting stage, allowing to distinguish between this and the progressive phases (Petzold et al., 2002). In line with these results, our group, in collaboration with Jack van Horssen (VU University, Amsterdam, The Netherland) demonstrated that S100B is overexpressed both in active

and chronic lesions mainly by astrocytes. While in active lesions S100B surrounds the demyelinated area, in chronic ones S100B is diffusely expressed within the demyelinated areas. Expression of S100B receptor RAGE was also shown to be exacerbated in active MS lesions, being expressed by macrophages/microglia.

S100B was also shown to be increased in CSF and serum of MS patients in both RRMS and progressive stages, levels that significantly decreased after therapy with an immunosuppressive agent (Bartosik-Psujek et al., 2011; Petzold et al., 2002). Accordingly, recent data from ongoing work of our group in collaboration with João Cerqueira (ICVS, Univ. Minho) demonstrated that in CSF samples from RRMS patients there is a significant increase of S100B production at the time of diagnosis. These results suggest that S100B might be a potential biomarker for MS diagnosis and prognosis allowing the distinction of different MS stages. On this background we hypothesize that S100B might play a role in the course of demyelination and remyelination events regarding MS.

3. Experimental Demyelinating Models

The lack of an effective therapy for the treatment of MS to date as well as the unknown of its correct pathophysiology led to the development of experimental models that mimic both the symptoms and the hallmarks of the disease. Rodents, such as rat and mice, have been a preferential target to develop human disease models, as they are easy to manipulate but also because of their relative genetically closeness to humans (Craig et al., 2003). The heterogeneity of MS causes it to be difficult to represent, leading to the development new models that allow the study of mechanisms of the disease towards its treatment (Murta and Ferrari, 2013).

3.1. *In vivo* Animal Models

One of the most widely applied models in MS research is experimental autoimmune encephalomyelitis (EAE). This model was developed in the late 20s as an

effort to understand neuroparalytic accidents, a complication of vaccination against rabies virus. Animals, initially non-human primates, were injected with brain tissue homogenates containing adjuvants, similarly to what was being used for rabies vaccination, and it was observed the occurrence of allergic encephalitis in result of such inoculations. Experiments were extended to other animals such as pigs, dogs, rats and mice and soon it was found a resemblance between EAE and MS histopathology (Croxford et al., 2011; Jervis, 1954; Waksman and Adams, 1962). Currently, EAE is mostly produced in C57BL/6 mice with the disease being induced by: (i) inoculation with myelin oligodendroglial glycoprotein (MOG) emulsified in an immunopotentiator solution (Freund's adjuvant supplemented with *Mycobacterium tuberculosis*); and (ii) and injection of pertussis toxin on the day of immunization and 2 days thereafter, which will stimulate cell-mediate immune response (Paterson, 1979). Although it has been demonstrated that EAE model plays a key role in the understanding of autoimmunity, neuroinflammation, cytokine biology and immunogenetics of MS, it presents some limitations (Waksman, 1999). In fact, EAE does not reproduce relapses which difficults the study of remyelination. There are still some differences between EAE and MS, such as the regions of the CNS that are mostly affected (spinal cord white matter and cerebral for EAE and cerebellar cortex for MS) and the cells of the innate immune response that are mostly activated (CD4+ for EAE and CD8+ T cells for MS) (Ransohoff, 2012; Saxena et al., 2011).

In order to fulfill some gaps presented by EAE model such as the lack of remyelination, an important hallmark of RRMS, there was a need to develop other models. An alternative *in vivo* model that showed to reproduce MS pathology was the administration of neurotoxins (Rodriguez, 2007). One of the most common toxin used is the copper chelator cuprizone that is fed with chow preferentially to vulnerable strains of animals (mice, rats) during 4-6 weeks, after which extensive remyelination ensues. Pathologically, cuprizone has demonstrated to cause mitochondrial dysfunction and to be selectively toxic to mature OL (Komoly et al., 1987; Venturini, 1973). One of the main advantages of cuprizone toxic model is the resemblance of demyelination and remyelination lesions to those occurring in MS patients, allowing a

closer insight into the disease itself and the expansion of treatment options for the patients (Denic et al., 2011; Ransohoff, 2012).

3.2. *Ex vivo* Organotypic Slice Cultures

In vivo models represent an expensive cost, both economically and ethically; thus, alternatives should be preferentially used whenever possible. Organotypic slice cultures have been used for decades in diverse CNS research fields, including electrophysiology and drug screening, mainly due to the maintenance of the three-dimensional architecture that held the tissues together allowing cell-cell interactions, and to their long-term survival in culture (Gähwiler, 1988; Stoppini et al., 1991).

Regarding MS, cerebellar organotypic slice cultures (COSC) have been shown to mimic demyelination and inflammatory processes when exposed to the toxin lysolecithin or lysophosphatidylcholine (LPC) (Allt et al., 1988). This toxin was primarily used to induce demyelination in a mice *in vivo* model by sub-perineurial injection on mature myelinated peripheral nerve fibers. Demyelination capacity was proposed to occur due to toxic detergent effects of LPC on myelin sheath (Hall and Gregson, 1971). Also, LPC-induced demyelination is thought to occur through the recruitment of macrophages and microglia which phagocyte the nearby myelin (Munder and Modolell, 1979).

The strength of this model when compared to *in vitro* models (i.e. cultures of OL and OPC), is the presence of the other glial cells, microglia and astrocytes, and neurons. This presence is a key factor as axons are needed for myelin ensheathment by OL, and also because astrocytes and microglia secrete factors that might promote or prevent myelination, accordingly to the surrounding environment (Dean et al., 2011; Schnädelbach et al., 2001). Moreover, the fact that the three-dimensional cell structure is relatively preserved is an essential aid for the occurrence of these events, as relationships among the different cell types are maintained (Avossa et al., 2003; Ghoumari et al., 2003). Furthermore, cerebellum is preferentially used in research studies regarding MS rather than other CNS regions, such as the spinal cord and the

brain stem, due to the abundance of white matter. Also, when submitted to LPC toxic stimulus, cerebellum requires less 2 hours of incubation than spinal cord and brain stem, which together allow a clearer perception of the lesions and to obtain faster results (Zhang et al., 2011a).

4. Aims

The main purpose of this work is to assess whether S100B is expressed in the course of a demyelinating insult and in what way its expression affects demyelination and remyelination, as well as reactivity of glial cells. For this, we will use a COSC from wild-type (WT) CD1 mice as model and induce demyelination insults using LPC.

In a first approach we will keep COSC for 7 days in culture, at which demyelination will be induced. COSC will then be maintained in culture for more 48 hours after the insult. At this point, we will determine S100B gene expression and release to the extracellular space, and which cells are producing it.

At a second approach we will incubate COSC with an anti-S100B antibody that will block S100B protein, in order to assess whether S100B is affecting demyelination and in what extent. We will also evaluate the reactivity of glial cells in response to the demyelinating insult, and in what way S100B might be involved in their activation.

II. . Material and Methods

1. Animals

Pregnant CD1 mice were acquired from Instituto de Higiene e Medicina Tropical (IHMT, Lisboa, Portugal). Animals were supplied with standard laboratory chow and water *ad libitum*. Animal care followed the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to use alternatives to *in vivo* techniques.

2. Organotypic Cerebellar Slice Cultures and Treatment

Parasagittal slices were obtained from cerebellum of CD1 mouse pups at postnatal day 10 (Figure II. 1). In brief, brains were removed, cerebellum and attached hindbrain were isolated in phosphate buffered saline (PBS) and 400 µm slices were obtained using a Mcllwain tissue chopper and kept in an air-liquid interface system. Separated slices were placed in the upper chamber of a 0.4 µm pore cell culture (BD Falcon, #353493, Lincoln Park, NJ, USA) in a number of 4 slices per insert. Cell culture inserts were maintained in 6-well cell culture plates containing 1 mL of medium in the plate well at a 37ºC and 5% CO_2 conditioned atmosphere. Slice culture media consisted of 50% minimal essential media (MEM, Gibco, Life Technologies, Inc.,Grand Islands,

USA), 25% heat-inactivated horse serum (Gibco), 25% Earl's balanced salt solution (Gibco), 6,5 mg/mL glucose, 25mM HEPES (Biochrom AG, Berlin, Germany) and 1% of both L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and penicillin/streptomycin (Sigma-Aldrich). After 3 DIV, slice culture media was totally replaced by a serum-free media consisting of 98% Neurobasal-A (Gibco) and 2% B-27 (Gibco), supplemented with 2 mM L-glutamine, 36 mM glucose, 1% U/mL penicillin/streptomycin and 25 mM HEPES. Half media was replaced every day and slices were maintained for 7 days *in vitro* (DIV) before treatment, to allow myelination and the clearance of debris (Birgbauer et al., 2004).

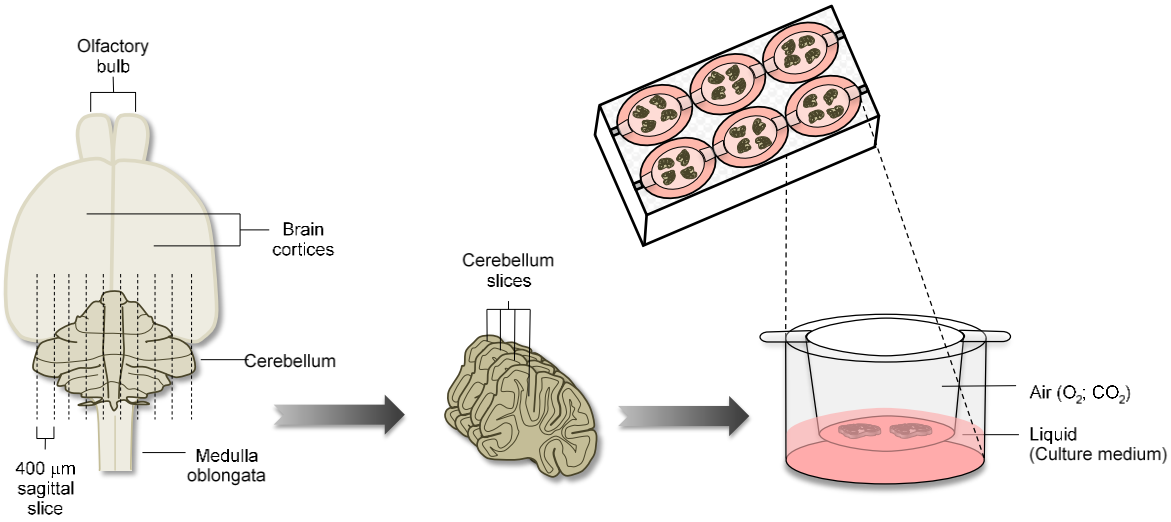

Figure II. 1 – Schematic representation of COSC protocol. Cerebellum, separated from the brain of CD1 mouse pups at postnatal day 10, are sectioned into 400 µm thick slices. Separated slices are placed into the upper chamber of a cell culture insert and kept in an air-liquid interface system.

Following the 7 days, COSC were exposed to a demyelinating insult with LPC (0,5 mg/mL in serum-free culture media (Sigma-Aldrich). Following 16h LPC, COSC were transferred to serum-free media in which cultures were maintained up to 48h (Birgbauer et al., 2004; Miron et al., 2010). In parallel experiments, to ascertain S100B role on demyelination and glial reactivity, COSC were incubated with LPC in the in the presence or absence of anti-S100B antibody (Figure II.2) (1:1000, #Z0311, Dako, Glostrup, Denmark). Supernatants were collected before and after LPC treatment. Slices were collected at 9 DIV (48h post-LPC), and either stored in TRIzol® reagent at -20ºC for RNA extraction or fixed in 4% paraformaldehyde (PFA) in PBS for 1h, rinsed

in PBS for 10 min twice and stored in PBS at 4ºC for immunohistochemistry assays.

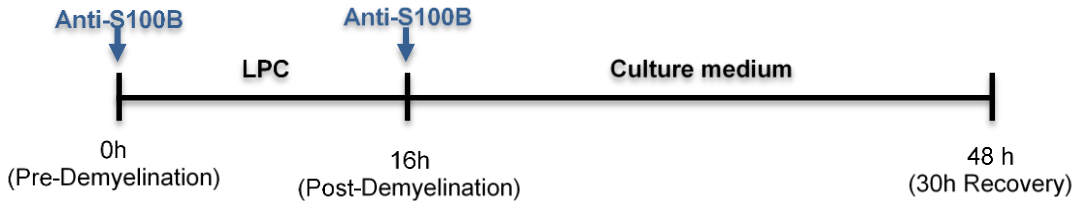

Figure II. 2 – Schematic representation of culture treatment. 0h, 16h and 48h correspond to 7 DIV, 8DIV and 9 DIV, respectively. LPC, lysophosphatidylcholine.

3. Total RNA Extraction, Reverse Transcription and Semi-quantitative Real-Time Polymerase Chain Reaction

In order to determine expression of S100B and other genes of interest, total cytoplasmic RNA was isolated from 9 DIV slices using the TRIzol® reagent method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA concentration was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 800 ng of total RNA were reversely transcribed using the Rivertaid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA), under recommended conditions. qRT-PCR was performed on a real-time PCR detection system (Applied Biosystems 7300 Fast Real-time PCR System, Applied Biosystem, Madrid, Spain) using a SYBR Green qRT-PCR Master Mix (Thermo Fisher Scientific, MA, USA). The PCR was performed in 8-well strips with each sample performed in duplicate, and a non-template control (NTC) was included for each amplificate. The sequences used as primers are listed in the Table II. 1

Table II. 1 – List of pairs of primers used for qRT-PCR assays. All primers were purchased from Thermo Fisher Scientific, MA, USA.

Gene	Sense	Anti-Sense
β-actin	gctccggcatgtgcaa	aggatcttcatgaggtagt
S100B	gagagagggtgacaagcacaa	ggccataaactcctggaagtc
MBP	ccatccaagaagaccccaca	cccctgtcaccgctaaagaa
PLP	tggcgactacaagaccacca	gacacacccgctccaaagaa
HMGB1	ctcagagaggtggaagaccatgt	gggatgtaggttttcatttctctttc
IL-18	tggttccatgctttctggactcct	ttcctgggccaagaggaagtg

qRT-PCR was performed under optimized conditions: 50ºC for 2 min, 95ºC for 10 min followed by 40 cycles at 95ºC for 15 s and 62ºC for 1min. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol (95ºC for 15 s, followed by 60ºC for 30s and 95ºC for 15s). Relative mRNA concentrations were calculated using the Pfaffl modification of the $\Delta\Delta C_T$ equation, where C_T is the cycle number at which fluorescence passes the threshold level of detection, taking into account the efficiencies of individual genes. The results were normalized to the housekeeping gene β-actin in the same sample and the initial amount of the template of each trial was determined as relative expression by the formula $2^{-\Delta\Delta CT}$. ΔC_T is the value obtained for each sample by performing the difference between the mean C_T value of each gene of interest and the mean C_T value of β-actin. $\Delta\Delta C_T$ of one sample is the difference between its ΔC_T value and the ΔCT of the sample chosen as reference.

4. Immunostaining procedure

In order to determine the location of S100B expression as well myelination impairment, membranes containing the fixed slices were cut out from cell culture inserts, placed onto a slide and blocked in 1nM HEPES (Sigma-Aldrich), 2% heat-inactivated horse serum, 10% heat-inactivated goat serum, 1% BSA (Sigma-Aldrich) and 0,25% Triton X100 (Roche Diagnostics, Indianapolis, USA) in Hank's Balanced Salt Solution (HBSS, Gibco) for three hours, at room temperature. Slices were then incubated with (Table II. 2) primary antibodies diluted in the blocking solution, for 24h at 4ºC. Following this, slices were washed three times for 15 min each with PBS with 0.01% Triton X100 (PBS-T) before incubation with secondary antibodies (Table II. 3) in blocking solution for another 24 h at 4ºC). Slices were then washed three times for 15 min each with PBS-T, incubated with DAPI (1:1000, 3 min), washed three times for 15 min each with PBS-T and mounted using Fluoromount-G (Southern Biotech, Birmingham, AL) for confocal microscopy.

Percentage of the area immunoreactive for each antibody was measured from 8 bit.lsm files of 512x512 pixel resolution images captured using a 20x/1.2(zoom) lens on a Confocal Point Scanning Microscope Zeiss LSM 510 META. Approximately, 4-5 images were captured per slice per condition, thus reducing any variations in image acquisition. Binary masks were defined using a cut-off intensity threshold value, defined as the minimum intensity due to specific staining above background values. Then, the percentage of the area occupied by NF200, S100B, MBP, GFAP, NG2 and Iba-1 staining was measured automatically for each separated stack of every acquired image using ImageJ software. Regarding myelination, percentage of myelinated fibers was obtained by the ratio between the area of co-localization of NF-200 and MBP and the total area occupied by NF-200. Moreover, percentage of total area occupied by myelin was calculated by the average of MBP area of each co-staining (with NF-200, NG2, GFAP and Iba-1). Results are given by averaging values determined in the separate microscopic fields from slices of different animals. Values are expressed as mean ± SEM.

Table II. 2 – List of primary antibodies used for immunoshistochemistry assays.

Antibody	Host	Brand	Category Number	Diluiton
S100	Rabbit	Dako	Z0311	1:400
NF-200	Mouse	Novocastra	NF200	1:200
MBP	Rat	Serotec	MCA409S	1:50
GFAP	Mouse	Novocastra	GFAP-GA5	1:100
Iba-1	Rabbit	Wako	019-19741	1:250
NG2	Rabbit	Milipore	AB5320	1:50

Table II. 3 – List of secondary antibodies used for immunoshistochemistry assays.

Antibody	G	Brand	Category Number	Diluiton
Alexa 488 anti-mouse	Goat	Invitrogen	A10680	1:1000
Alexa 488 anti-rabbit	Goat	Invitrogen	A11008	1:1000
Alexa 594 anti-rat	Donkey	Invitrogen	A21209	1:1000
Alexa 594 anti-rabbit	Goat	Invitrogen	A11001	1:1000

5. S100B assay

Determination of S100B concentration released to the supernatants was performed by in house enzyme-linked immunosorbent assay (ELISA). Samples were incubated for 2 h at 37ºC on a 96 well plate (NUNC, Kamstrup, DK-4000 Roskilde, Denmark) previously coated with a monoclonal anti-S100B antibody (1:1000, #S2532, Sigma-Aldrich) 96-well plate previously coated with a monoclonal anti-S100B antibody (1:1000). Next, a polyclonal anti-S100B antibody (1:5000, Dako) was added and plate incubated for more 30 min at 37ºC. Finally, an anti-rabbit peroxidase-conjugated antibody (1:5000, #sc-2004, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added for further 30 min at 37ºC. Colorimetric reaction with Sigma Fast OPD tablets® (Sigma-Aldrich) was measured at 492 nm in a BioRad microplate absorbance spectrophotometer.

6. Statistical Analysis

All results are presented as mean ± SEM. Differences between two groups were determined by the two-tailed t-test performed on the basis of equal and unequal variance or by one-way ANOVA with Tukey post-test for multiple comparisons, using GraphPad PRISM 5.0 (GraphPad Software, San Diego, CA, USA), as appropriate. The *P*-values of $P<0.05$ and $P<0.01$ were considered as being statistically significant.

III. Results

1. S100B is overexpressed and released from central nervous system cells following demyelination insult

As previously mentioned, abnormal levels of S100B have been detected in the CSF and in *post mortem* plaques of MS patients (Bartosik-Psujek et al., 2011; Petzold et al., 2002). Thus, we wanted to attest weather S100B was being expressed in our model of demyelination. For this purpose, the levels of S100B protein released to the extracellular space were quantified in culture or incubation media collected before the incubation with LPC (0h), at 16h and 48h following the LPC stimulus by ELISA. As shown in Figure III. 1A, a marked increase in the release of S100B occurred at 16 h after LPC incubation (20.8-fold over control, $P<0.001$), which was maintained 48h following the LPC incubation (8.5-fold over control, $P<0.001$).

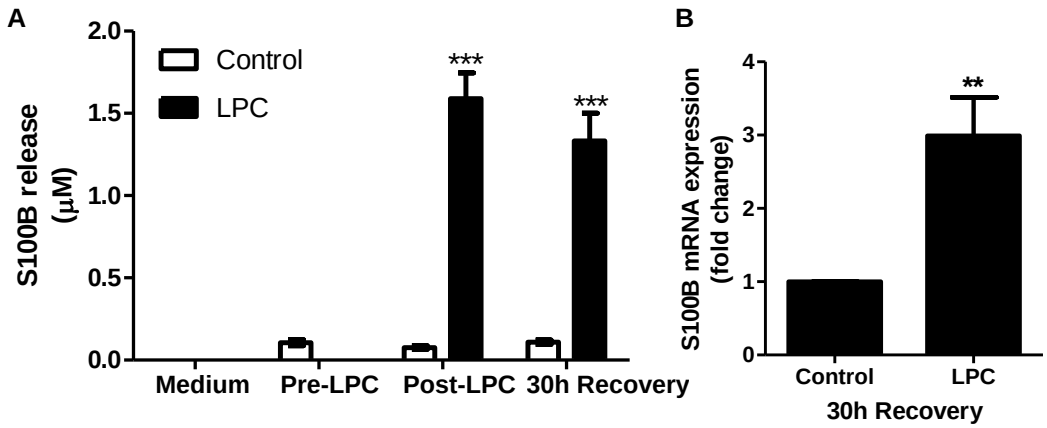

Figure III. 1 – A demyelinating insult induces S100B production. Cerebellar organotypic slice cultures (COSC) were exposed to lysophosphatidylcholine (LPC) at 7 days *in vitro* (DIV) (0 hours) for 16 hours. Samples for detection of S100B secretion were collected before the incubation (0h), at 16h and 48h following the incubation with LPC (A). Samples for analysis of mRNA expression were collected 48 after the LPC stimulus (B). Results are mean ± SEM. ***P*<0.01, ***P<0.001 vs Control.

In addition, we also measured the expression of S100B mRNA in COSC at 48h following LPC insult by qRT-PCR. Using primers specific to the S100B mouse gene we showed a significant increase of S100B expression in response to the demyelinating insult (3-fold over control, $P<0.01$) (Figure III. 1B).

2. S100B is mainly released by astrocytes during demyelination

It is known that in the CNS S100B is expressed by most cells, and mainly secreted by astrocytes (Van Eldik and Zimmer, 1987). Knowing that S100B is being overexpressed and released to the extracellular space following the LPC insult, we wanted to assess which cells were generating such effect. For this end, we have compared the percentage of co-localization between S100B and neurons, astrocytes or OL. COSC fixed 48h following LPC insult were double immunostained with antibodies against S100B and one type of cell marker NF-200, GFAP or MBP which allow the identification of neuronal axons, astrocytes and mature OL, respectively.

As show in Figure III. 2, there is a major co-localization of S100B with GFAP (55.8%), even in control samples. Upon LPC treatment, co-localization of S100B with GFAP is still the most prevalent (37.5%), although we could observe a slight increase of S100B and MBP co-localization. These results allow us to assume that astrocytes are the major producers of S100B under demyelinating circumstances, although it is possible that mature OL may also be releasing S100B under this toxic condition, as previously documented (Steiner et al., 2008a).

3. Blockade of S100B partially attenuates demyelination induced by LPC

Knowing that S100B is being overly secreted in response LPC-induced demyelination, we wanted to find out whether this increase in extracellular S100B could affect myelination related events. For this end, we evaluated the percentage of myelinated fibers in COSC at 48h post-LPC insult.

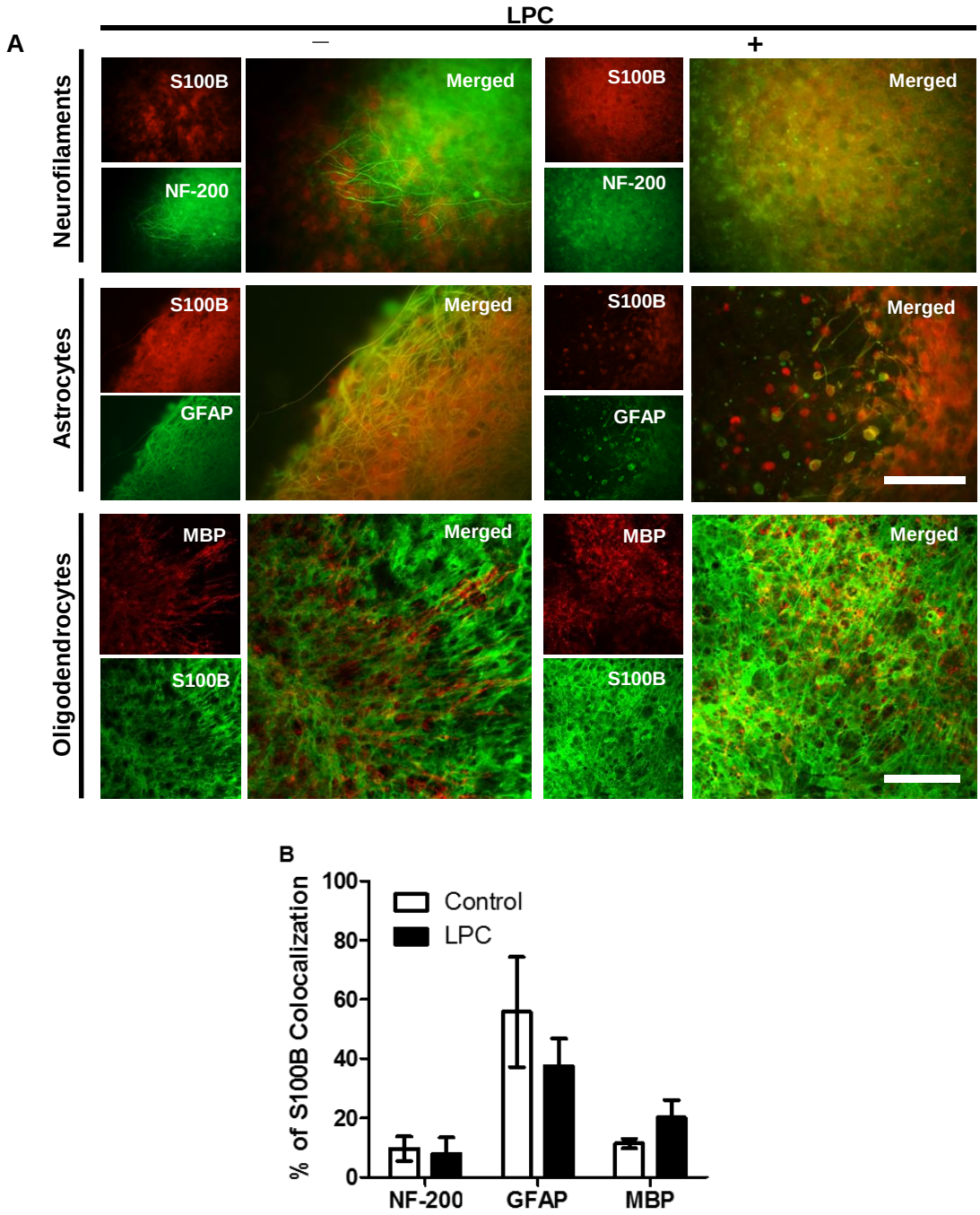

Figure III. 2 - S100B is mainly released from astrocytes upon demyelination. Cerebellar organotypic slice cultures (COSC) were exposed to lysophosphatidylcholine (LPC) at 7 days in vitro (DIV) (0 hours) for 16 hours. Immunostainings were performed in PFA fixed COSC 48 hours post-LPC, with S100B, NF-200, GFAP and MBP antibodies (A). Quantification of the percentage of co-localization between S100B and neurons, astrocytes and OL was measured by the ratio between the area occupied by S100B and NF, GFAP or MBP staining and the area occupied by S100B staining (B). Scale bars represents 100 μm. Results are mean ± SEM.

Slices were double immunostained with antibodies against both NF-200 and MBP, which allow the identification of neurofilaments and myelin sheaths, respectively. The percentage of myelinated fibers was calculated by the ratio of the area of co-localization of NF-200 and MBP and the area occupied by NF-200. As depicted in the Figure III. 3, LPC stimulus effectively damaged myelin sheaths, which is corroborated by the decrease observed in MBP staining (0.8 -fold) and in myelinated fibers (0.6-fold, $P<0.01$). Interestingly, co-incubation with anti-S100B antibody partially decreased the demyelinating insult caused by LPC stimulus as observed by a reduced decreased in MBP staining (0.9-fold) and in myelinated fibers (0.8-fold) over control values. (Figure III. 3A). The effect on myelinated fibers was significant, indicating that S100B inhibition may prevent demyelination.

As previously mentioned, MBP and PLP are the main proteins constituents of myelin sheaths (Colman et al., 1982). As so, we decided to evaluate the expression of these proteins under demyelinating conditions caused by LPC incubation and how S100B could be affecting their expression. For this purpose, we measured the mRNA of MBP and PLP extracted from COSC 48h after LPC insult. The analysis of MBP and PLP mouse genes was performed by qRT-PCR with primers specific to the mouse genes. As illustrated in Figure III. 5, LPC insult promoted a significant increase in MBP and PLP expression (3.94-fold and 3.79-fold over control, respectively, $P<0.01$). Interestingly, co-incubation with anti-S100B antibody decreased the expression of both genes to a level close to the control expression (1.34-fold for MBP and 2.05-fold for PLP, over control).

In general, demyelinating events are followed by spontaneous remyelination, which involves recruitment and proliferation of endogenous OPC into the injured area (Gensert and Goldman, 1997; Zhao et al., 2005). Thus, given the effective demyelination caused by LPC, we then aimed to explore whether remyelination was succeeding demyelination through the evaluation of the presence of OPC. For this purpose, COSC fixed 48h following LPC insult were double immunostained with antibodies against NG2, for OPC and against MBP for mature OL.

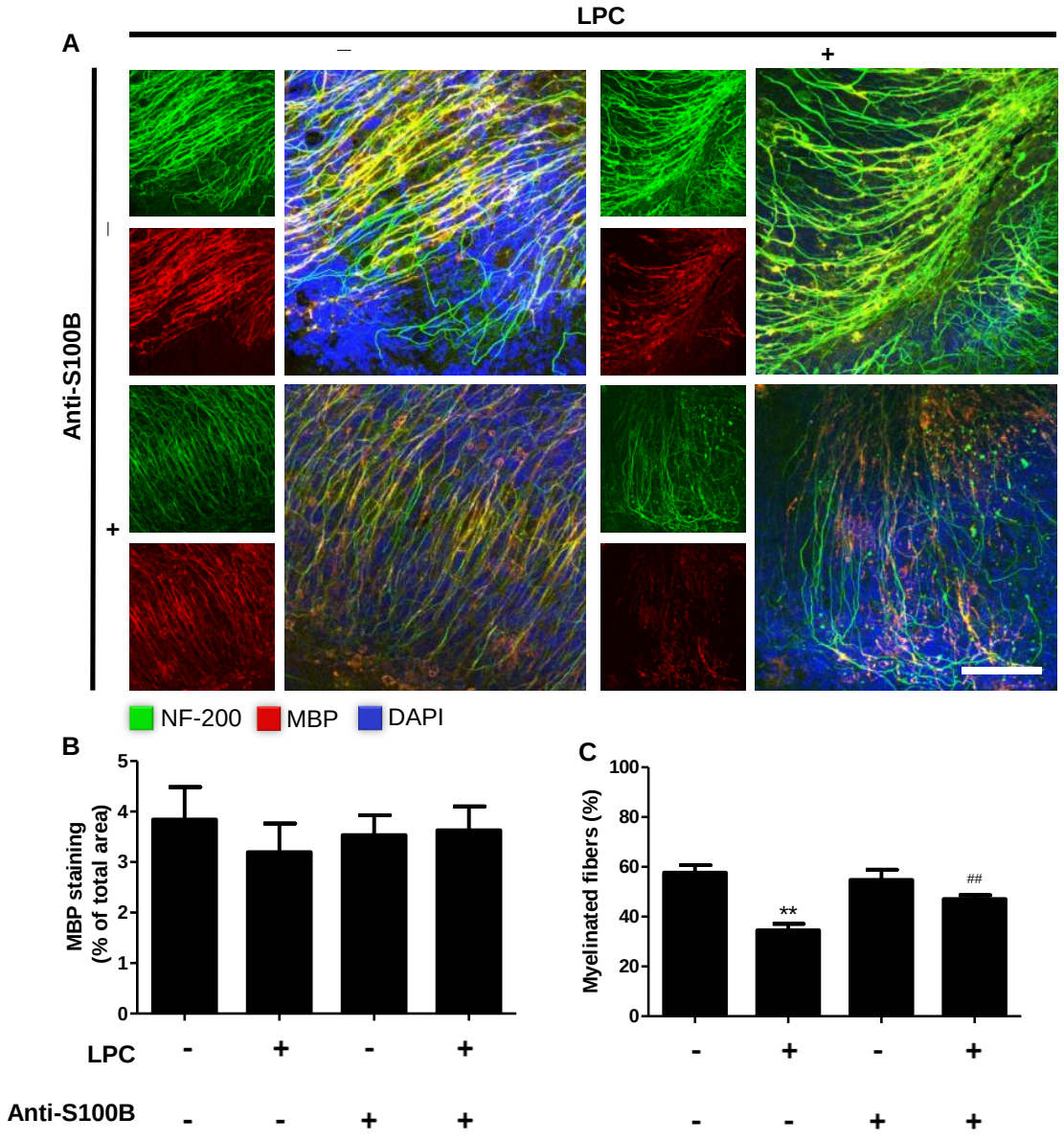

Figure III. 3 – Antibody directed blockade of S100B partially attenuates demyelination caused by LPC. Cerebellar organotypic slice cultures (COSC) were exposed to lysophosphatidylcholine (LPC) at 7 days in vitro (DIV) (0 hours) for 16 hours. Double imunostainings were performed in fixed COSC at 48h post-LPC with NF-200 and MBP antibodies (A). Area occupied with MBP staining was obtained by averaging the percentage of MBP area of each co-staining (B). Quantification of the percentage of myelinated fibers was calculated by the ratio between the area of co-localization of NF-200 and MBP and the total area occupied by NF-200 (C). Results are mean ± SEM. **P<0.01 vs control. ##P<0.01 vs LPC alone. Scale bar represents 100 μm.

The total area of OPC was calculated by averaging the percentage of area occupied by NG2 staining for each stack. As depicted in Figure III.5, LPC demyelination insult caused an increase in the number of NG2 positive cells (1.9 fold, *P*<0.05). Yet, co-incubation with anti-S100B antibody showed to enhance this effect (3.0-fold, *P*<0.01),

while the addition of anti-S100B by itself can stimulate NG2+ cells proliferation. These data suggest that the presence of low levels of extracellular S100B, not bound to the anti-S100B antibody, might be exerting a recruiter and/or proliferative effect on OPC.

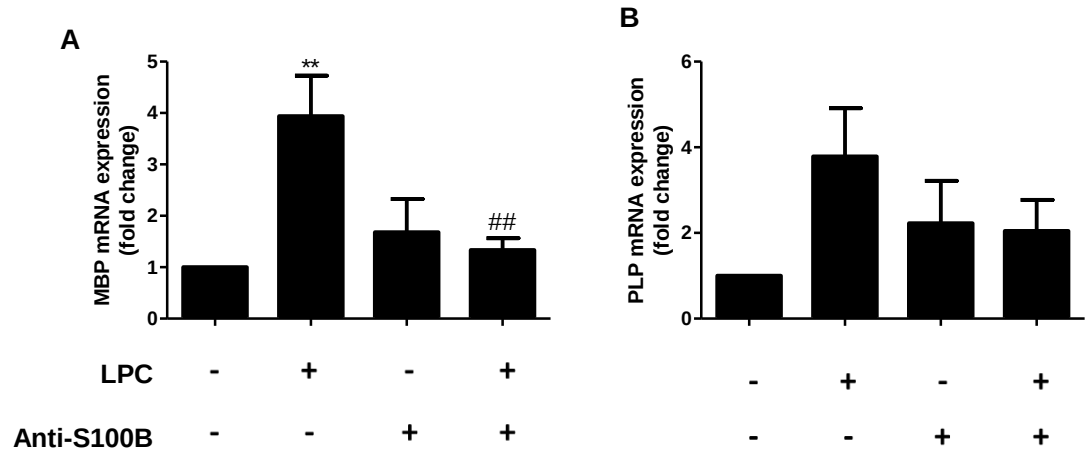

Figure III. 4 – Blocking S100B decreases the expression of myelin genes following demyelination. Cerebellar organotypic slice cultures (COSC) were exposed to lysophosphatidylcholine (LPC) at 7 days in vitro (DIV) (0 hours) for 16 hours. Total mRNA was extracted from COSC at 48h post-LPC and gene expression was analyzed by qRT-PCR with primers specific to the MBP and PLP mouse genes. Results are mean ± SEM. **P<0.01 vs Control. ##P<0.01 vs LPC alone.

4. Abrogation of S100B decreases glial reactivity provoked by demyelation

Along with myelin degeneration, demyelinating lesions are known to be succeeded by activation and proliferation of resident astrocytes and microglia (Dyer et al., 2005; Sofroniew and Vinters, 2010). Regarding this, and knowing that S100B is mainly secreted by astrocytes and is involved in the activation of both astrocyte and microglia, we decided to evaluate the degree of reactive gliosis in COSC in the course of the demyelinating insult with LPC, and what would be the potential role of S100B in such events.

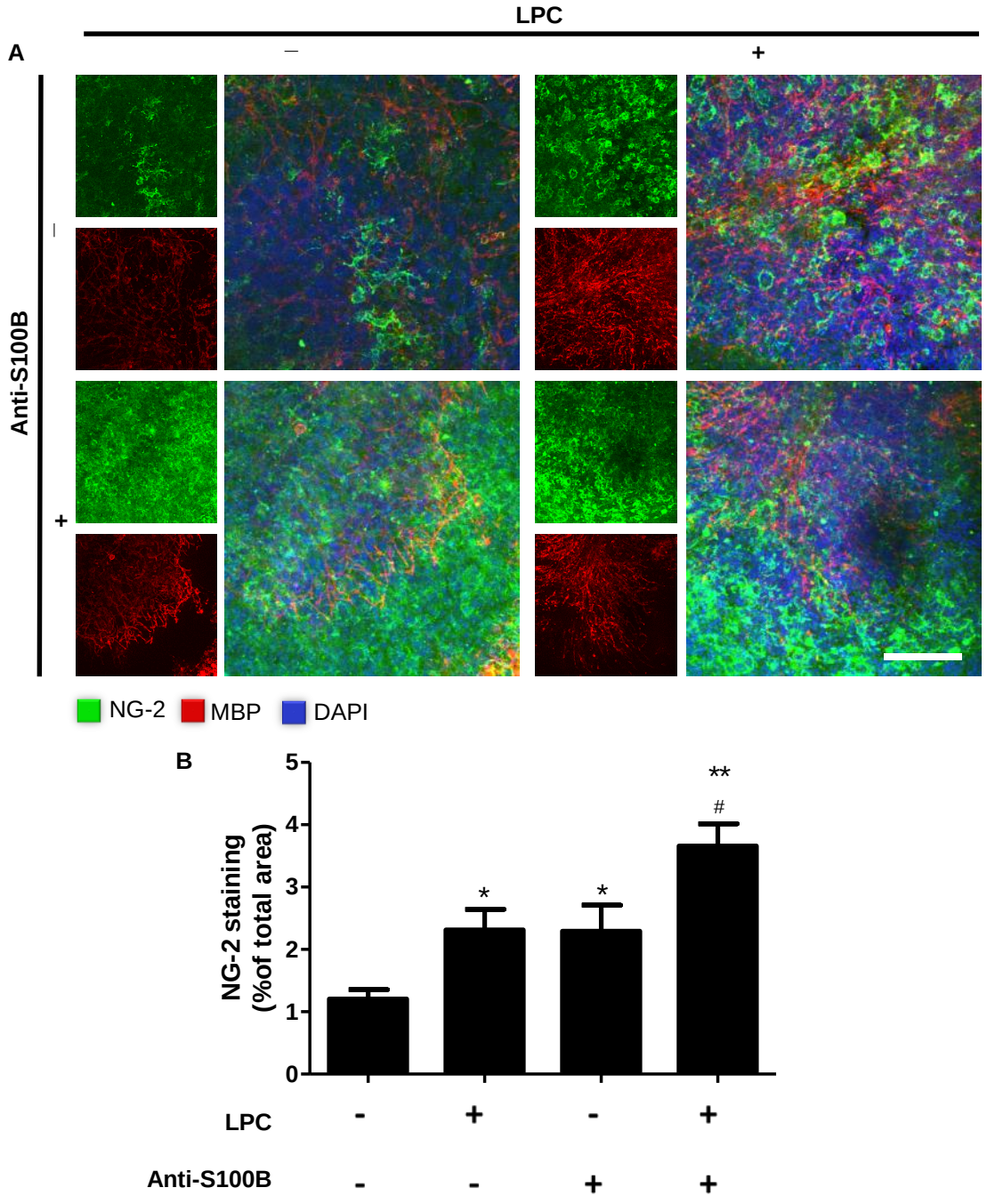

Figure III. 5 – S100B abrogation causes an increase in OPC activation. Cerebellar organotypic slice cultures (COSC) were exposed to lysophosphatidylcholine (LPC) at 7 days in vitro (DIV) (0 hours) for 16h. Immunostainings were performed in fixed COSC at 48h post-LPC, with MBP and NG2 antibodies (A). Quantification of the area occupied by OPC was measured as the average of the area occupied by NG2 staining for each stack (B). Results are mean ± SEM. *P<0.05, **P<0.01 vs control; #P<0.05 vs LPC alone. Scale bar represents 100 μm.

For this end, we evaluated the proportion of astrocytes and their morphology by double immunostaining of fixed COSC at 48h after LPC insult with antibodies for both MBP and GFAP, a well-established marker of astrogliosis. As shown in Figure III.6, LPC stimulus decreased the percentage of area occupied by astrocytes (0.4-fold, $P<0.01$ vs control), as a consequence of reduced extension number and length presented by activated cells. Interestingly, when COSC were co-incubated with anti-S100B antibody we observed an increase in the percentage of the area occupied by astrocytes (1.2-fold vs control) to control levels, indicating a reduced activation state as evidenced by a more ramified morphology.

Regarding microglia, we evaluated the proportion of microglia and their morphology by double immunostaining of fixed COSC at 48h following LPC insult with antibodies for both MBP and Iba-1, a commonly used marker for microgliosis. As shown in Figure III.7, incubation with LPC revealed to increase the percentage of Iba-1 positive cells (2.0-fold, $P<0.01$). In turn, co-incubation with anti-S100B antibody in the presence of LPC maintained the levels of microglia found for LPC treatment (1.9-fold, $P<0.01$). Nevertheless, confocal imaging suggested that microglia position in slice could be altered. So, we explored the distribution of microglia along the slice, by normalizing the slice thickness for the amount of myelin and dividing into four equal percentiles. Indeed, the bottom and top of the slice were defined by the presence of MBP staining, the lowest stack showed reduced percentage of MBP area corresponding to the bottom of the slice (0) whereas the highest percentage corresponds to the top of the slice (1) (Figure III. 8B). Interestingly, we observed a growing percentage of the area occupied by Iba-1 staining along the slice, upon incubation with LPC, namely the bottom half. Co-incubation with anti-S100B did not prevent increased Iba-1 staining, but this increase was more evident in the upper half of the slice corresponding to the region with highest amount of myelin (Figure III. 8 A,C). These data suggest that in the presence of low S100B (i.e. the amount free from anti-S100B binding) microglia presents a tendency to migrate into demyelinated lesions, possibly to increase the phagocytosis of myelin debris, a feature that is crucial for remyelination.

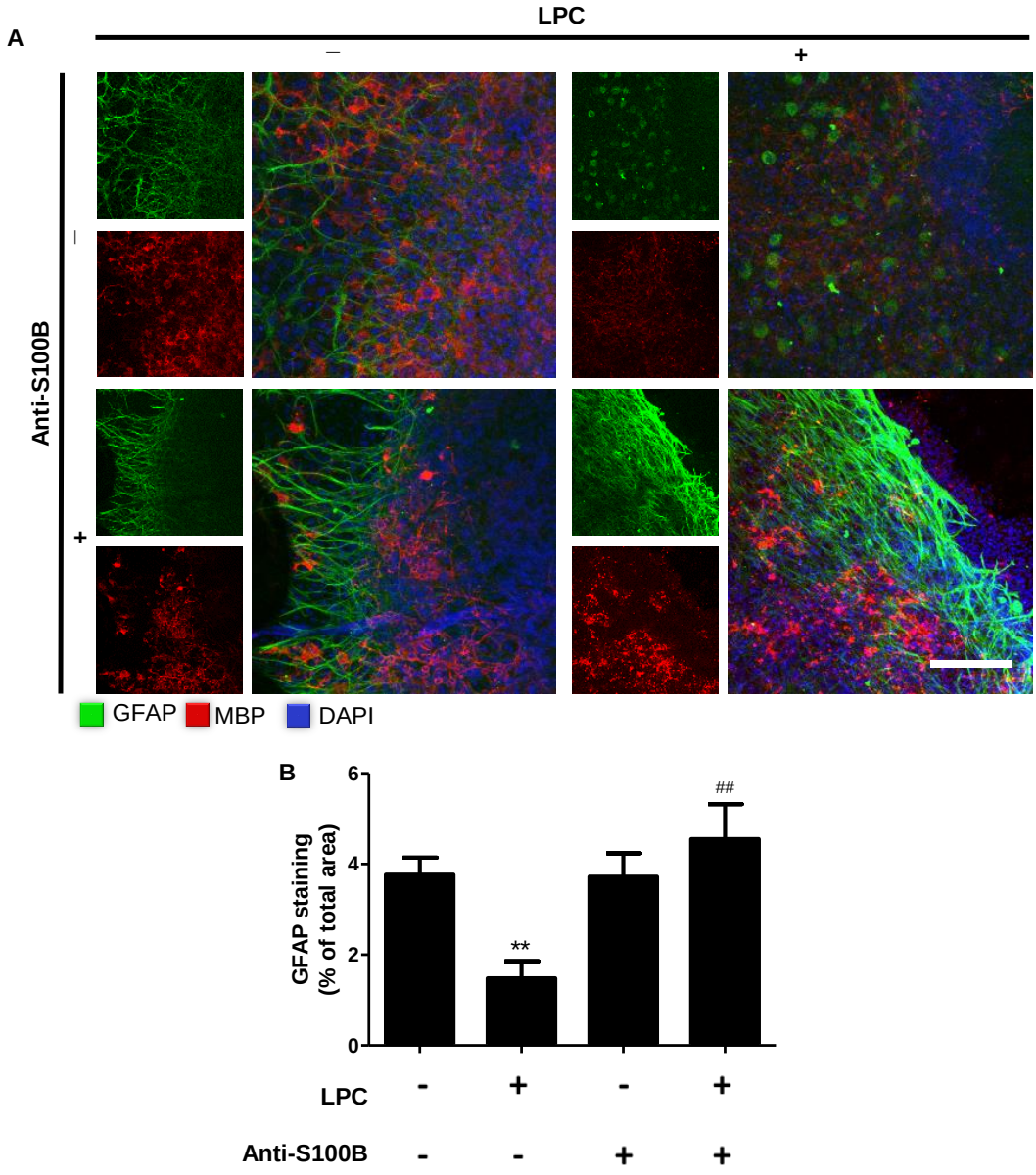

Figure III. 6 – S100B blocking prevents astrocytic activation triggered by demyelination. Cerebellar organotypic slice cultures (COSC) were exposed to lysophosphatidylcholine(LPC) at 7 days in vitro (DIV) (0 hours) for 16h. Immunostainings were performed in fixed COSC at 48h post-LPC, with GFAP and MBP antibodies (A). Quantification of astrocytes was taken by averaging the area occupied by GFAP staining for each stack (B). Total mRNA was extracted from COSC 48h post incubation with LPC and the analysis of qRT-PCR products illustrated was conducted with primers specific to GFAP mouse gene (C). Results are mean ± SEM. **P<0.01 vs control, ##P<0.01 vs LPC alone.. Scale bar represents 100 μm.

Altogether these results suggest that low levels of S100B might play a protective role under demyelinating conditions as it appears to revert astrocyte and

microglia activation, possibly attenuating the trigger of inflammatory response and creating an environment that favors remyelination.

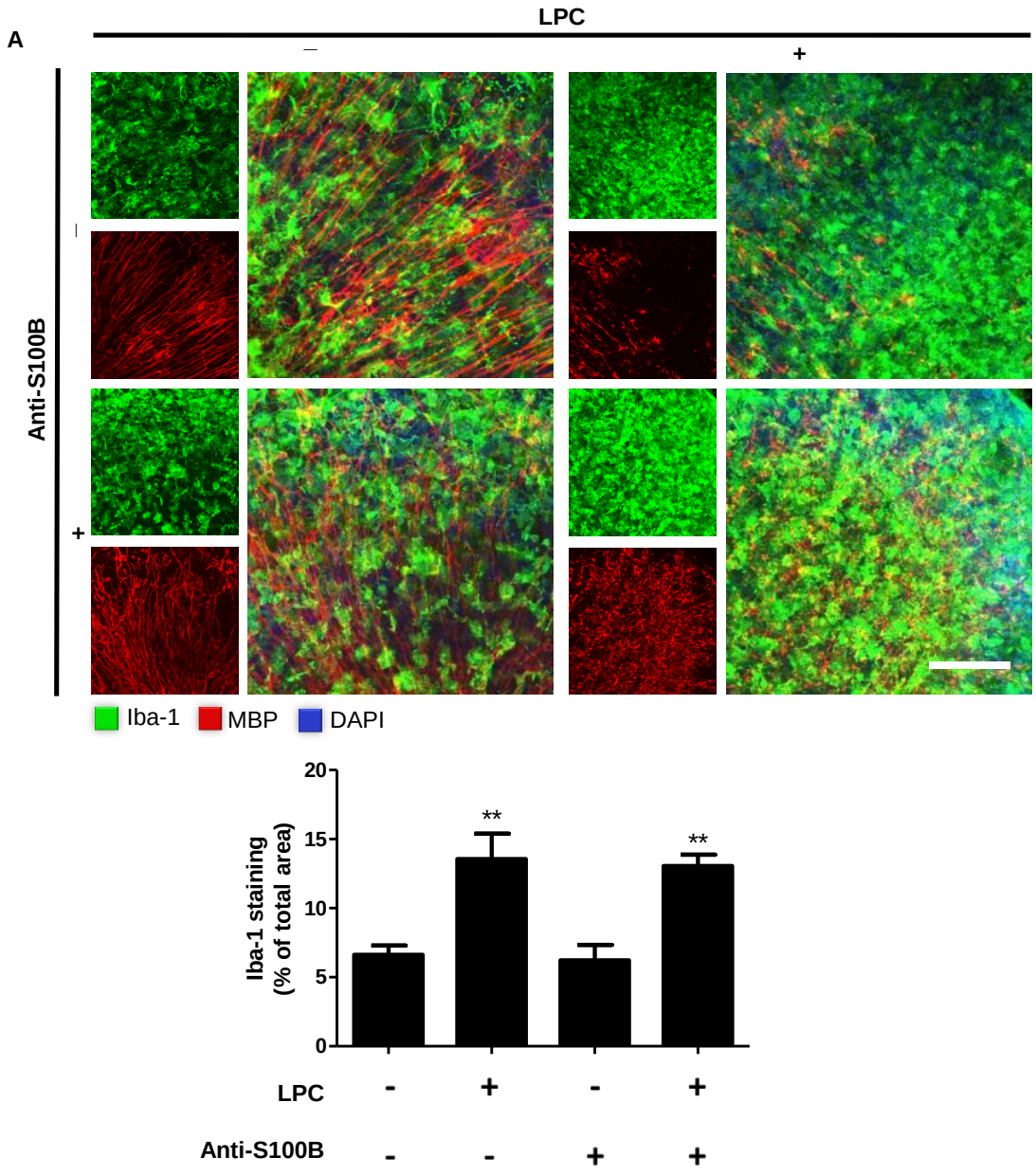

Figure III. 7 –S100B blocking partially attenuates microglia activation induced by LPC demyelination. Cerebellar organtypic slice cultures (COSC) were exposed to lysophosphatidylcholine (LPC) at 7 days in vitro (DIV) (0 hours) for 16h. Double immunostainings were performed in fixed COSC at 48h post-LPC, with Iba-1 and MBP antibodies (A). Quantification of microglia was taken by averaging the area occupied by Iba-1 staining for each stack (B). Total mRNA was extracted from COSC 48h post incubation with LPC and the analysis of qRT-PCR products illustrated was conducted with primers specific to cd11b mouse gene (C). Results are mean ± SEM. **P<0.01 vs Control. Scale bar represents 100 μm.

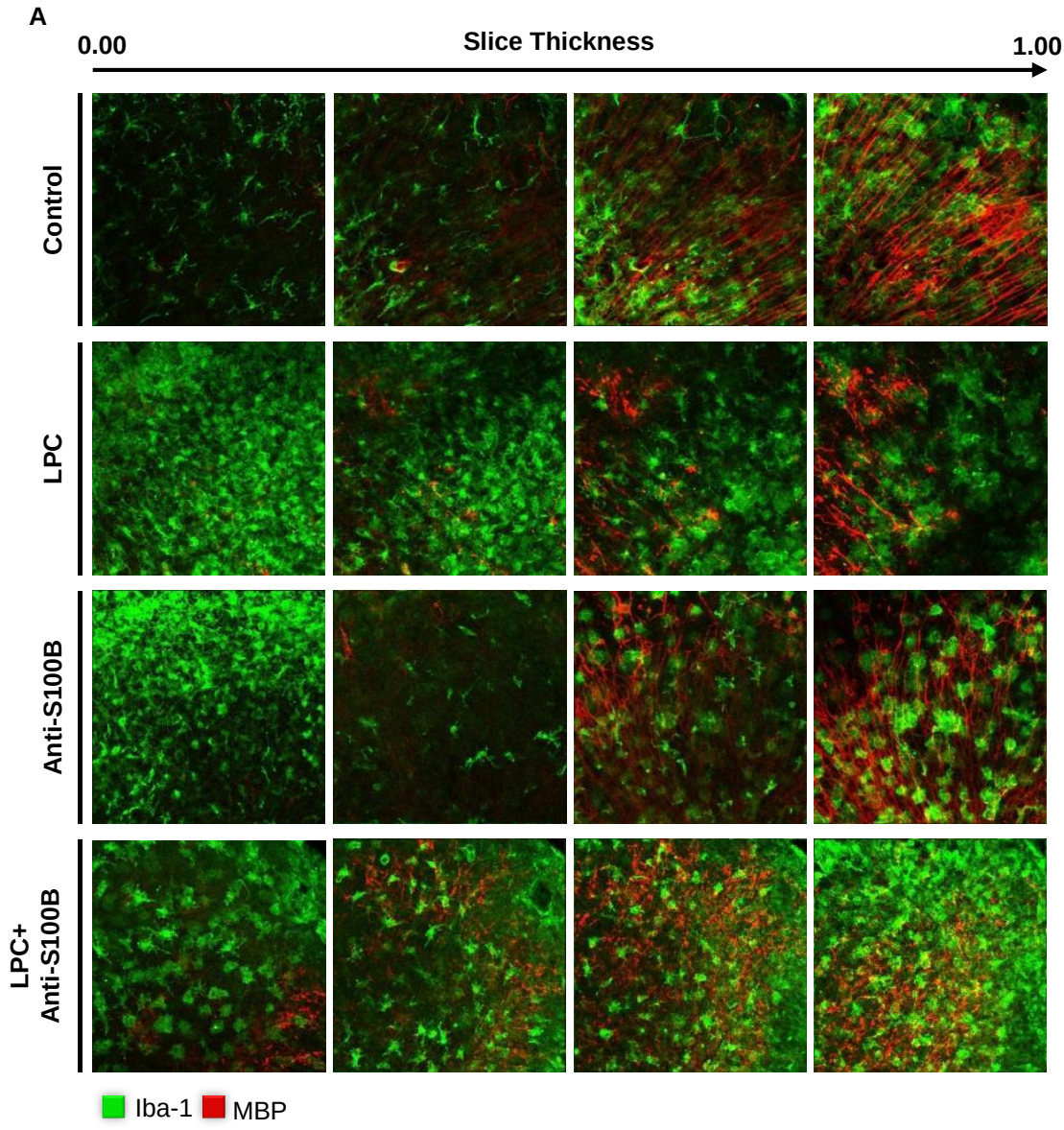
A
0.00
Slice Thickness
1.00
Control
LPC
Anti-S100B
LPC+
Anti-S100B
Iba-1
MBP
B
1.00
0.00
C
Iba-1 staining
(% of total area)
20
15
10
5
0
0,25
0,50
0,75
1,00
Slice thickness
Control
LPC
Anti-S100B
LPC+Anti-S100B

Figure III. 8 – Blocking S100B apparently induces clearance of debris by microglia following demyelination. Cerebellar organtypic slice cultures (COSC) were exposed to lysophosphatidylcholine (LPC) at 7 days in vitro (DIV) (0 hours) for 16h. Double immunostainings were performed in fixed COSC at 48h post-LPC, with Iba-1 and MBP antibodies (A). Slice thickness was normalized for MBP staining, with 0 corresponding to the bottom of the slice (lowest MBP immunofluorescence) and 1 to the top of the slice (highest MBP immunofluorescence) (B). Normalized slice thickness was divided into four percentiles with the same relative thickness, and the area occupied by Iba-1 staining was analyzed for each percentile (C).

5. Inflammatory response triggered by demyelination is attenuated by S100B blockade

Along with astrocytic and microglial activation, demyelination is also characterized by an exacerbated production of pro-inflammatory cytokines and chemokines (Sospedra and Martin, 2005). Since previous studies have shown that HMGB1 and IL-18 are upregulated in MS patients (Andersson et al., 2008; Chen et al., 2012), we intended to evaluate the expression of these cytokines. For this end, we extracted total mRNA from COSC at 48h following LPC insult and HMGB1 and IL-18 mRNA expression was analyzed by qRT-PCR using specific primers. As shown in Figure III. 9, LPC insult promoted a marked increase in the expression of both genes (14.76-fold for HMGB1 and 10.87-fold for IL-18, over control).

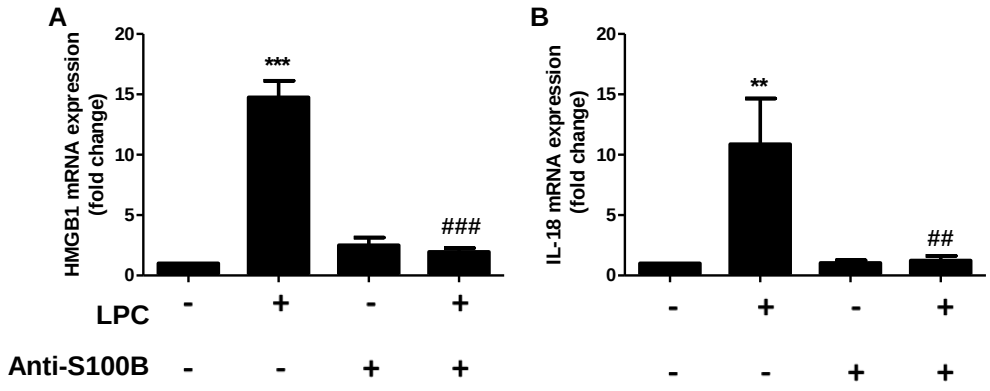

Figure III. 9 – S100B abrogation significantly decreases expression of pro-inflammatory cytokines HMGB1 and IL-18 elicited by demyelination. Cerebellar organotypic slice cultures (COSC) were exposed to lysophosphatidylcholine (LPC) at 7 days in vitro (DIV) (0 hours) for 16h. Total mRNA was extracted from COSC 48h at post-LPC and HMGB1 and IL-18 mRNA expression was analyzes by qRT-PCR using primers specific to HMGB1 and IL-18 mouse genes. Results are mean ± SEM. Results are mean ± SEM. **P<0.01, ***P<0.001 vs Control. ##P<0.01, ###P<0.001 vs LPC.

Additionally, when co-incubated with anti-S100B antibody, the expression of both HMGB1 and IL-18 significantly decreased to levels similar to control (1.95-fold for HMGB1 and 1.25-fold for IL-18, over control). These results clearly suggest that the by reducing S100B to physiological levels we might be somehow preventing the initiation of the inflammatory response.

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IV. Discussion

S100B has currently been target of many studies related to neurodegenerative disorders. Indeed, abnormal levels of S100B expression have been detected in both CSF and *post-mortem* plaques of MS patients. The major hallmark of MS, an autoimmune demyelinating chronic disease of the CNS, is the formation of sclerotic plaques resultant from a process that involves reactive gliosis, inflammation, demyelination and remyelination, OL depletion, axonal loss and neuronal degeneration (Milo and Miller, 2014). Moreover, as previously mentioned, S100B has been described as being a mediator of inflammatory response in brain injury conditions (Donato et al., 2009).

With this work we intended to study the role of S100B in MS associated mechanisms, using a COSC model of demyelination (Birgbauer et al., 2004), addressing in particular demyelination and inflammation-related events. Even though abnormal levels of S100B have been detected in MS patients, there is no record of its direct involvement in demyelination mechanisms, so far. First of all, we evaluated both the production and expression of S100B in the course of the demyelinating insult with LPC. Our results revealed an overproduction and overexpression of S100B with a major co-localization with astrocytes, rather than neurons, and a slight co-localization with mature OL, detected by immunostaining directed to these cells (Figure III. 2). These data suggest that, in the model used in our work, astrocytes are the major producers of S100B in the course of demyelination. In fact, astrocytes are assumed as the CNS cell type with the highest expression rate of S100B and to constitutively secrete the protein (Donato, 2001; Van Eldik and Zimmer, 1987). In agreement, recent studies in rat COSC have shown an increase in astrocytes population 2 DIV after a

demyelination insult with LPC (Miron et al., 2010). The small co-localization with OL may suggest a reduced secretion of S100B by these cells when exposed to a toxic stimulus, as previously described for OL-93 cell line under serum and glucose deprivation conditions (Steiner et al., 2008b)

Accordingly to previous reports, we observed that the concentration of LPC used as well as the length of the stimulus were effective in inducing demyelination, demonstrated by a reduction in the number of myelinated fibers (Figure III. 3) (Allt et al., 1988; Birgbauer et al., 2004). Interestingly, we observed that by adding an anti-S100B antibody to the incubation media in the presence of LPC, that can partially sequester the excessive release of S100, myelin sheaths damage was prevented. Additionally, the expression of the major proteins of myelin MPB and PLP was markedly enhanced following LPC injury. Previous studies on different *in vivo* models of demyelination have documented that the expression of MBP and PLP decrease immediately after the insult but undergo *de novo* synthesis afterwards, forecasting remyelination (Lindner et al., 2008; Woodruff and Franklin, 1999). These data appear to be in accordance with our results, since we observed a greater expression of MBP and PLP mRNA 30h after LPC incubation, relative to the control, suggesting an initiation of remyelination. Nevertheless, treatment with anti-S100B antibody abrogated MBP or PLP *de novo* synthesis when compared to LPC incubation.

MBP and PLP *de novo* expression is necessary for remyelination (Lindner et al., 2008; Zhang, 2001). Studies on COSC show that OPC differentiation and maturation as well as re-expression of myelin proteins occurs in a proportional temporal sequence than what would be observed *in vivo* (Birgbauer et al., 2004). As mentioned above, blockade of S100B is preventing extended demyelination by retrieving concentrations close to physiological. This way, it is possible that by no major need of remyelination, OL and*de novo* synthesis of myelin proteins might be reduced, as depicted in our results. In addition, we may not neglect the fact that MBP or PLP mRNA expression may be occurring at different time-points from the one-analyzed.

Remyelination requires activation of resident of OPC that will migrate to the injured area, proliferate and maturate into myelinating OL (Gensert and Goldman, 1997). The activation of OPC is characterized by a morphological change, where they assume a larger size and become more intensely labelled with NG2 (Kotter et al., 2001; Levine and Reynolds, 1999). Accordingly, our results reveal an increase in OPC activation following a transient demyelinating insult with LPC, shown by an augmented area of NG2+ cells.. Preliminary data from ongoing work in our group shows that pathological extracellular concentrations of S100B (1000 nM) affect oligodendroglial lineage development, specifically preventing differentiation of OPC, what may justify sich increase. Nevertheless, blocking of S100B in the media increased even more the levels of NG2+ OPC, suggesting a higher induction of the cells to a remyelinating event. It is known that S100 is necessary for OPC differentiation into mature OL (Deloulme et al., 2004; Zhang, 2001). As so, it is possible that the presence of small levels of S100B that are not sequestered by the antibody may be promoting OPC proliferation for consequently differentiation and remyelination.given these data, additional studies should be performed for more extended periods of culture in order to analyze the remyelination potential of anti-S100B treatment.

Reactive gliosis has long been associated with MS pathophysiology (Compston and Coles, 2008). Reactive astrocytes, which are implicated in formation of the glial scar, are characterized by dramatic morphological (cellular hypertrophy) and gene regulation (upregulation of GFAP) changes (Sofroniew and Vinters, 2010). Increased astrocytosis has also been observed during LPC induced demyelination in an COSC model (Miron et al., 2010). Our results show an increase of astrocytic activation upon LPC transient demyelination insult, evidenced by the reduction of cell extension lenght and inflated cell body. This activation was shown to be subsided with incubation of anti-S100B. As previously discussed, astrocytes abundantly secrete S100B when exposed to the demyelinating agent. Moreover, excess of extracellular S100B levels induce autocrine astrocytic activation. In this situation, astrocytes secrete pro-inflammatory factors that can inhibit OPC proliferation and maturation, following demyelination in MS (Cammer and Zhang, 1999). Thus, the apparent absence of astrocytosis under

demyelinating conditions in the presence of anti-S100B, is a potential indicative that low levels of S100B might be preventing demyelination. Moreover, astrocytes in response to demyelination, under a non-inflammatory environment, are also responsible for the production of growth factors and chemokines that enhance OPC activation and consequently their migration and differentiation (Blakemore et al., 2003). As so, the reduced activation of astrocytes induced by anti-S100B antibody treatment may also corroborate the augmented number of NG2+ cells.

Microglia activation in MS pathophysiology contributes to the creation of a pro-inflammatory environment, by the secretion of pro-inflammatory factors, potentiating demyelination. Microglia activation has also been described in COSC models as consequence of demyelination (Birgbauer et al., 2004; Miron et al., 2010). Accordingly, our results reveal an increase in microglia proliferation and activation in the course of demyelinating insult, depicted by an augmented percentage of Iba-1 staining and apparent morphological change. Since S100B concentration on the range of μM, as the ones measured in the present study, induce micoglia secretion of pro-inflammatory cytokines (Adami et al., 2001; Donato et al., 2009), it may be possible that S100B-induced microglia activation exacerbates demyelination.

On the other hand, our results showed that blocking of S100B did notmicroglia activation, although its presence shifted to myelin surrounds, as observed when different Z-stacks were analyzed (Figure III. 8). Microglia have an important role in the trigger of remyelination, as it promotes the clearance of myelin debris (Kotter et al., 2001; Napoli and Neumann, 2010). In fact, there is a recent evidence that microglia switch from a cytotoxic to protector phenotype at remyelination initiation (Miron et al., 2013). Moreover, extracellular S100B is known to inhibit microglia activation at physiological concentrations (Adami et al., 2001; Donato et al., 2009). As so, our results suggest that the low extracellular levels of S100B (i.e., not bound to anti-S100B antibody) under LPC plus anti-S100B treatment might be preventing microglia from acquiring a cytotoxic phenotype and promoting their phagocytosis and clearance of myelin debris, which will favor remyelination.

As previously referred, MS pathophysiology holds an inflammatory background. Particularly, activation of astrocytes and microglia in response to demyelination involve the secretion of several factors, such as pro-inflammatory cytokines. HMGB1, a cytokine involved in the initiation of inflammatory response within CNS, has recently been demonstrated to be upregulated in both acute MS lesions and rodent EAE lesions (Andersson et al., 2008). Moreover, IL-18, which also participates in the pathogenesis of autoimmune diseases as MS, has been shown to be increased in MS patients (Chen et al., 2012; Huang et al., 2004). In accordance, our results revealed a significant increase in the expression of both cytokines following LPC stimulus. interestingly, blockade of S100B showed to effectively revert the expression levels of these cytokines in a situation of demyelination. A recent report have pointed out that inhibition of IL-18 diminishes demyelination and promotes remyelination in a NLRP3 dependent manner (Jha et al., 2010). NLRP3, a member of the nucleotide-binding domain, leucine-rich repeat containing (NLR) family, pyrin-domain containing 3, is a core component of the inflamasome complex, and it has been proposed to be related to neuroinflammation and demyelination events (Sutterwala et al., 2006). Hence, it would be interesting to investigate the expression of NLRP3 in relation to S100B in our model of demyelination. In adition, since these cytokines as mainly expressed by activated microglia, their inhibition following S100B blocking corroborates microglia shift from a pro-inflammatory phenotype to a more neuroprotective one.

Concluding Remarks

With this work we demonstrated, in a COSC model, that LPC effectively induce demyelination and subsequent S100B overproduction. Moreover, the demyelinaton event led to the upregulation of myelin proteins and pro-inflammatory cytokines gene expression, as well as activation of astrocytes and microglia. In turn, blockade of pathological S100B levels appeared to prevent demyelination and promote increased remyelination, as well as to decrease both reactive gliosis and expression of pro-inflammatory factors. Altogether, our results strongly suggest that S100B might be

directly involved in demyelination and remyelination mechanisms, in a concentration dependent manner.

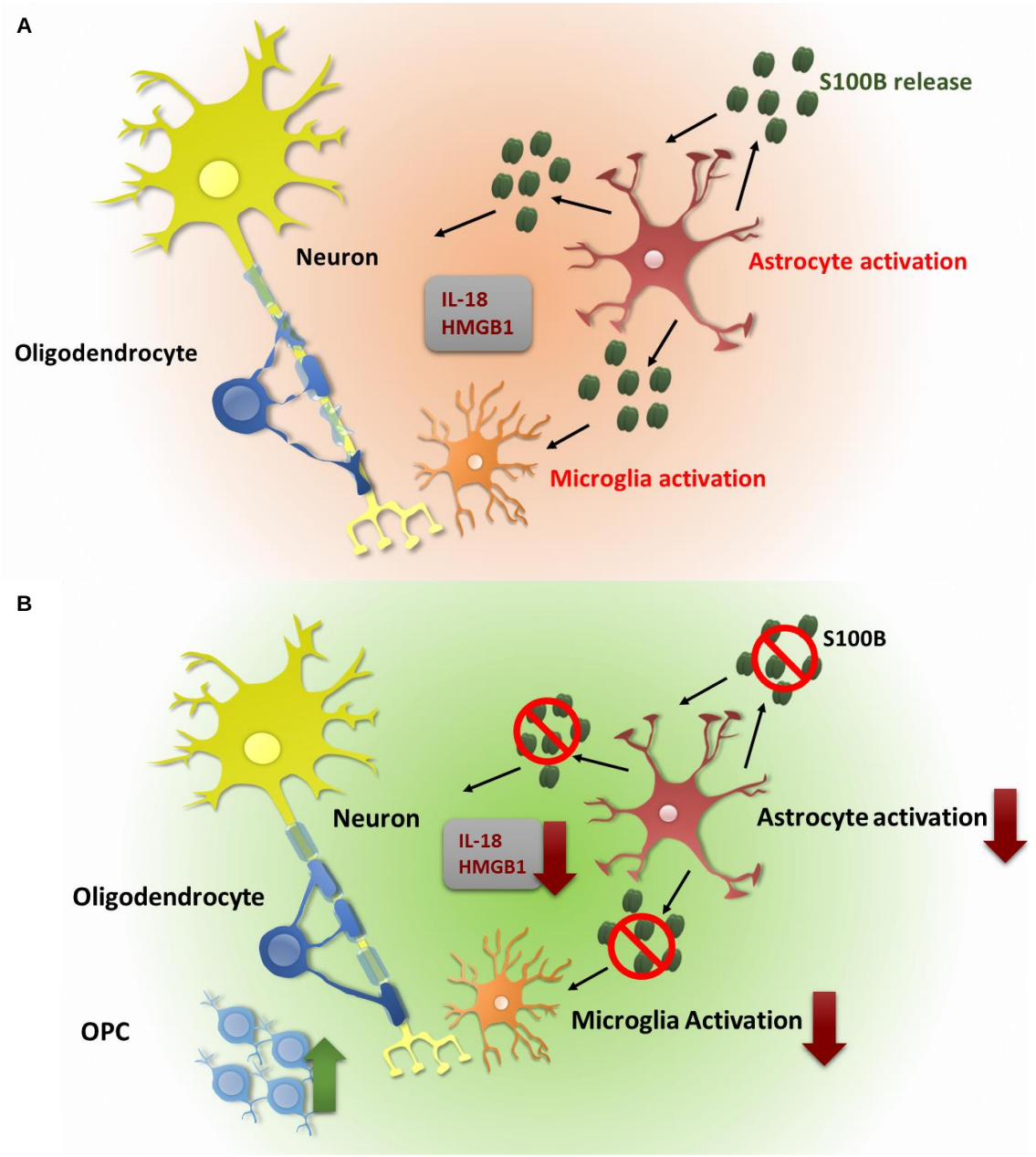

Figure IV. 1 – Schematic representation of main findings. (A) A situation of demyelination showed to induce secretion of S100B (mainly by astrocytes), microglia and astrocytes activation, and upregulation of pro-inflammatory cytokines. (B) Blocking S100B revealed to revert astrocyte activation and cytokine production, change microglia to a more neuroprotective role and to increase the number of OPC, as well.

V. References

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